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HPV NEL DISTRETTO TESTA COLLO:

SCREENING MOLECOLARE PER UNA VALUTAZIONE EPIDEMIOLOGICA SU CAMPIONI ITALIANI.

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SUMMARY

Oral carcinoma is the most common of malignant tumors of the cavity oral and is still a serious problem for human health with an impact clinical in terms of incidence, prevalence and mortality rates that does not tend to improve. Diagnosis is often made when the malignancy is advanced and consequently the prognosis is poor with high morbidity and mortality. High-risk human papilloma viruses (HPV) types 16 and 18 as aetiological agents of anogenital carcinoma have been firmly established in the literature. Because of the morphological similarities and epitheliotropic nature of HPV, a link between OSSC and HPV seemed logical and has been the focus of numerous studies. The relationship between HPV and the oral mucosa has been supported by several investigators reporting the presence of HPV-DNA in healthy oral mucosa.

During the first year of attendance at the Pathology and Oral Medicine Outpatient Clinic of the Cà Granda IRCCS Hospital in Milan, the design bases were laid for a search for HPV serotypes particularly related to the possible predisposition and onset of oral lesion muco-cutaneous and oral and oro-pharyngeal neoplasms.

The second part of the study contemplate the recruitment of subjects investigated through biopsy and histopathological examination for the diagnosis of HPV pathologies related to the mucous membranes of the oral cavity (squamous papilloma, acute condyloma, verruca vulgaris) attending Pathology and Oral Medicine Outpatient Clinic of the Ca'Granda IRCCS Hospital in Milan. 12-month recruitment period from October 2018 to October 2019. The intent of this initial study, which will progress beyond the conclusion of this path, is to evaluate a rapid, effective and achievable method on a large scale to extend the research of HPV even in patients not suffering from overt correctable HPV pathologies in order to plan a screening for the follow-up of the development of related HPV oral cancer genotyping in the study of cancer is useful for providing further data on the causal relationship between cancer and viral infection, on oncogenic HPVs and on the cellular modifications they induce. On the vaccinated population, it allows to monitor the duration and effectiveness of protection and is

useful in stratifying HPV positive women and men based on the risk of developing precancerous or cancerous lesions and thus to guide clinical decisions. The genotyping tests in use today differ in their analytical capabilities for sensitivity and specific type specificity.

In the vaccination era, a high analytical sensitivity is required, as a failure to identify the prevalent infections upon entry into a trial can lead to a false result of vaccine failure in vaccination protocols.

Chapter 1

ORAL SQUAMOUS CELL CARCINOMA

Chapter 1

Oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is the eighth most common malignancy in the developing world (1). High-risk human papilloma viruses (HPV) types 16 and 18 as aetiological agents of anogenital carcinoma have been firmly established in literature (2). Because of the morphological similarities (3) and epitheliotropic nature of HPV, a link between OSSC and HPV seemed logical and has been focused in numerous studies. The relationship between HPV and the oral mucosa has been supported by several investigators reporting the presence of HPV-DNA in healthy oral mucosa (4-6) A predilection of HPV for certain, especially non-keratinized anatomical sites in the oropharynx, was confimed when HPV16-related DNA sequences were detected in 16%, 51%,60% and 13% of SCC of the tongue, tonsil, Waldeyer's ring, and pharynx respectively (7–9). This resulted in the identfication of a distinct subset of head and neck SCC, particularly tonsil carcinoma, which have a strong and consistent association with high-risk HPV types with molecular characteristics indicative of viral oncogene function (10). HPV16 and 18 are the most widely implicated HPV types in the OSCC literature.

Oral cancer predominantly affects people of the fifth-sixth decade of life. However, in recent years the incidence of oral cancer in subjects under the age of 60 years has increased dramatically in the US and Europe.

Only a small percentage of patients have demonstrable distant metastases at presentation. For this reason, traditional therapy consisted of surgical resection with postoperative radiation (2). Although this approach is often effective in locoregional control of disease, there can be devastating consequences for personal appearance and critical functions such as speech and swallowing. Sixty to eighty percent of patients with early-stage disease achieve a curative result with modern therapy, and metachronous primary cancers of the upper aerodigestive tract have become a focus of current clinical studies, which emphasize chemoprevention strategies (3). For patients with stage III and IV disease,

long-term survival rates are generally low, ranging from 30 to 40% (2,4). As a result, intensive efforts are under way to develop efficacious combined-modality treatment programs, integrating chemotherapy with radiation (4–7). Objectives want to improve local and regional disease control, enhance survival, and maintain function. Rehabilitative therapy focusing on speech and swallowing, attention to dentition, and nutritional support have become routine therapeutic considerations (8,9). Longitudinal assessment of life quality measures also is now included in outcome analyses of head and neck cancer treatment programs.

The carcinogenic process is gradual and requires multiple genetic alterations in the epithelia of the upper airway. These genetic changes generate phenotypic alterations in tumor cells that allow them to continue to survive and expand. Mathematical models estimate that approximately seven to ten individual genetic alterations must occur for the development of cancer. Head and neck cancer is believed to originate via a multistep process that involves the activation of oncogenes and the inactivation of tumor suppressor genes. However, the specific pattern of progression and the genetic alterations necessary have not been delineated. Inactivated tumor suppressor genes and activated oncogenes, some identified and some inferred from tumor-specific chromosomal losses, have been the subjects of intensive study.(9)

Furthermore, it is hypothesized that some individuals are particularly susceptible to even limited environmental exposures.

Oral squamous cell carcinoma is characterized by an early infiltrative tendency and a major lymphatic spread compared to the hematogenous one. Lymph node infiltration is affected from the site of the tumor, from its size, from the histotype tumor, histological differentiation, anatomical structure of the affected organ and its lymphatic network, from relationships histopathological with the close anatomical formations. The diffusion lymph node can occur through the passage of tumor embolus primitive through the internodal lymphatic vessels or by diffusion direct extracapsular. The site of lymph node metastasis is in genus related to the site of the primary neoplasm. Distant metastases are

rare and late. The main office affected is the lung, rare localization to bones, liver and brain, exceptional other localizations.

Lymphatic diffusion is assessed through clinical examination palpatory, followed by imaging techniques (ultrasound, CT, MRI). Palpation of the neck has an extremely variable sensitivity (16-66%) depending on the ability and experience of the operator, of depth of the formations to be examined and the anatomy of the neck. Lymphatic diffusion is an almost constant and sometimes very event early and, with the exception of distant metastases, constitutes the single most important negative prognostic element. The frequency of lymph node involvement is correlated with the size and depth of invasion, thickness, location, degree of differentiation and duration. Lymphatic diffusion generally follows constant paths depending on the size and especially the site first involving the submandibular and submental lymph nodes. Primary tumors located in the midline metastasize bilaterally, while in the lateral sites the metastasis is in unilateral gender. (10)

1.1 Oral precancerosis lesions.

1.1.1Oral leucoplakia

Given the broad definition of oral leukoplakia, a wide variety of clinically apparent oral white lesions can meet the definition. Typically, oral leukoplakia begins as a slightly elevated white plaque that may have sharp or indistinct borders. (11) As the lesion matures and progresses, its thickness and white color often increase. Several categorizations have been described for oral leukoplakia to encourage some descriptive uniformity among providers. (12) One such categorization identifies lesions as either homogenous (having a uniform, flat appearance that may exhibit superficial irregularities but has a consistent texture) versus nonhomogenous (primarily irregular and may have red and white aspects to the lesion).(13) Oral leukoplakia with an intermixed red component has been referred to as speckled leukoplakia or mixed leukoplakia/erythroplakia. Leukoplakia can develop on any surface of the oral mucosa, with the most commonly described locations being the mandibular alveolus (25.2-40%), buccal mucosa (21.9-46%), palate (27%) or tongue (26%), and floor of

mouth (19.3%). Most patients present with multifocal disease. A clinically similar entity that can be confused with leukoplakia is frictional keratosis. This typically occurs at a site of chronic irritation or rubbing commonly because of a denture, tongue chewing, or cheek chewing. These lesions are clinically indistinguishable from leukoplakia; however, clinical history identifies the frictional source. These lesions should resolve with cessation of the mechanism of irritation. The clinical importance of oral leukoplakia derives almost entirely from its identity as a precursor to OCSCC. Long-term, observational, population-based studies have provided us with the best estimates of the likelihood of malignant transformation.

1.1.2 Proliferative verrucous leukoplakia

Proliferative verrucous leukoplakia (PVL) is an unusual form of oral leukoplakia that is typically multifocal and persistent, has a tendency to recur, and evolves into exophytic lesions that resemble verrucous carcinoma. Most importantly, PVL has a high risk for becoming dysplastic and transforming into squamous cell carcinoma (SCC). In one study of 30 patients with PVL, 86.7% of patients developed carcinoma at the lesion site at a mean time of 6 years. (14-15) In another series of 54 patients with PVL, most patients had multiple sites affected (mean 2.6) and the most common site was the buccal mucosa for women and the tongue for men.

1.1.3 Oral Candidosis

The relationship between candidiasis and oral squamous cell carcinoma remains debated. In fact, Candida spp infection and the risk of malignant transformation of potentially malignant disorders was first hypothesized time at the end of the 1960s by Cawson et al. (16). Since, numerous targeted epidemiological investigations have been conducted to explain of the role of the fungus in the progression process epithelial carcinomatous without, achieve results satisfactory and decisive. Among the clinical-histopathological forms of primary oral candidiasis, the one most investigated in this sense is chronic hyperplastic candidiasis.

It occurs in the form of a chronic lesion, with whitish plaques with faded edges, or strongly opaque and hardened, in the homogeneous or plaque-like variant with a white-greyish color on an erythematous underground mucosa.

1.1.4 Oral lichen planus

Oral Lichen planus (LPO) is an inflammatory mucocutaneous chronic disease with immune pathogenesis and relatively unknown ethology frequent and often localized only in the oral cavity. Italian data report a prevalence of 1.46% (6). It occurs clinically bilateral and symmetrical white striae (striae of Wickham) in the posterior third of the cheeks, on the tongue and gums. Other typical manifestations are white plaques leukoplasic-like and atrophic-erosive lesions: the latter are the true symptomatic lesions. The tongue is the most frequent site of cancer, women appear more affected and there is no difference of cancerization between white and red lichen.

1.1.5 **Hpv and OSCC**

Molecular and epidemiologic studies have demonstrated that a persistent infection with high-risk (HR) human papillomavirus (HPV) is a necessary risk factor for the development of cervical intraepithelial lesions and invasive carcinoma.

The infection can evolve, based on the genotype involved and the degree of cellular differentiation, in different ways:

a) latent infection: the viral genome undergoes replication in episomal form, being present as an extrachromosomal fragment of circular DNA duplicated a low number of times in the non-permissive basal cells (early replication);

b) productive infection: viral DNA is sequentially expressed, starting from early genes (E) to late ones (L), following differentiation in the squamous sense of the epithelium (vegetative replication).

In the upper layers of the epithelium (intermediate and superficial) there is a significant production of capsidic proteins and to the formation of the complete virion, an infecting viral particle, released

with desquamation of superficial keratinocytes;

c) transforming-persistent infection: sustained by capable HR HPVs to insert own nucleotide sequences in cell chromosomes in correspondence of common fragile sites. The insertion takes place through the breakdown of viral episomal DNA at regions of viral DNA with transcriptional suppressive action on the sequences viral oncogene (E5, E6 and E7) which, expressed in an exuberant way, subvert (by altering the action of different onco-suppressors) the normal growth mechanism of the epithelial cell causing uncontrolled proliferation and genetic instability. The prevalence of infection in carcinomas of the head-neck district seems to vary according to the location, being significantly higher in oropharyngeal carcinomas compared to oral squamous cell carcinoma, by virtue of the aforementioned increased anatomic susceptibility. A significant association between HPV infection and tonsillar carcinomas versus carcinomas oral is also confirmed by other investigations, which recognize in the virus an important risk factor for a large proportion of carcinomas of the oropharynx and, probably, for a small one proportion of oral carcinomas, and suggest a carcinogenic model specific for the oro-pharyngeal district alternative to that tobacco- and alcohol-associated.

1.2 Diagnosis

Biopsy consists of taking pathological tissue from an organism in order to study its microscopic characteristics end come to a diagnosis. Provides information on the nature of the injury and especially on the presence of dysplasia. In particular the incisional biopsy appears to be a reliable diagnostic tool in the interception of dysplasia and squamous carcinoma of the oral mucosa. Performing a biopsy sampling, particularly in the case of multiple withdrawals, although not involving particular difficulties of operating technique, presupposes a specialized competence. Concerning to PMDs there are two possible techniques to withdraw of the fragments in order to submit them to a histopathological examination: excisional or incisional biopsy. (17)

Excisional Biopsy consists in removing the entire lesion and then set up the histological examination on the operating piece to formulate the diagnosis. Excisional biopsy is indicated in suspicious lesions

of limited extension (<1cm) with much invasion superficial, as long as it is performed by an expert in cancer surgery oral, with the radical criteria adopted for a carcinoma. (18)

It is a medical procedure that is both diagnostic and therapeutic. The risk, not having yet a diagnosis of the lesion, is that of perform excisions or too large or too small; in both cases the error can be serious and, especially in the second, irreparable. The incisional biopsy on the other hand, consists in the sampling of one or more fragments of the suspected lesion, which therefore remains substantially unchanged and clearly identifiable pending future therapy. (19)

1.3 Therapy

Traditionally, oral cavity carcinomas in the initial stage are treated with surgery or radiotherapy, since the results obtained with the two methods would seem substantially overlapping.(20) Non-surgical therapies in these cases present the considerable advantage of allowing organ and limit the functional damage secondary to surgical procedures.(21) However, it should be noted that the significant progress achieved in the field of plastic and reconstructive surgery allow to minimize the disfiguring effects and loss of function imposed by the demolition surgery. (22) In advanced cancer the most effective response remains linked to surgical therapy, currently also applied in case of particularly extensive injuries. Surgical reconstruction must therefore be considered an indispensable phase of the intervention, since it is thanks to the high reconstructive potential that it is possible carry out effective demolition interventions in an oncological sense, (23) planning aesthetically and functionally satisfactory rehabilitation. In advanced lesions, radiation therapy is used with adjuvant purposes and enhancement of the surgical result, although associations have been proposed as an alternative to surgery of one or more chemotherapy drugs integrated into the therapy radiant. The relative therapeutic failure in terms survival indicates the need of study the effective multimodal therapeutic protocols for the application of new therapeutic strategies based on results research in the immunological, molecular, genetic and early diagnosis of cancer. (24)

Chapter 2

PAPILLOMAVIRUS

2.1 Introducing papillomavirus

Papillomavirus, originally included together with polyomaviruses in the Papovaviridae family for its common capsid and genomic characteristics, is currently officially recognized by the International Council on Taxonomy of Viruses (ICTV) as belonging to the Papillomaviridae family (25)

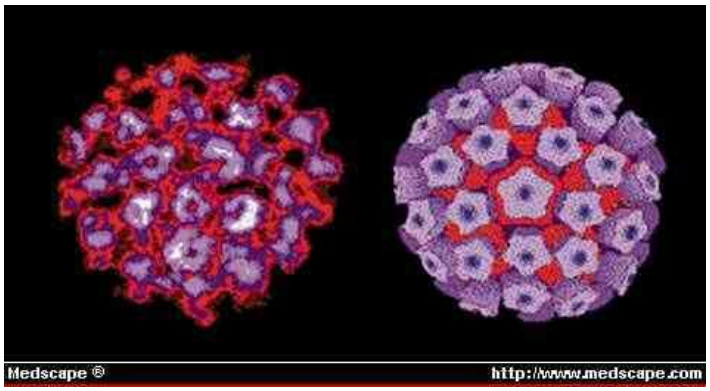

Fig.1: Computerized reconstruction of the three-dimensional structure of the human Papillomavirus (from: http://www.medscape.com)

Papillomaviruses are widespread in nature and can infect a large number of vertebrates, however having a very high species-specificity and a specific tropism for squamous epithelial cells.

HPV is a small virus (52-55 nm in diameter), the virion has no envelope; the icosahedral capsid is composed of 72 capsomers formed by two structural proteins L1 and L2; the genome is double stranded circular DNA (about 8,000 bp), the site of viral DNA replication and capsid assembly is nuclear. The papillomavirus genome has at least a dozen open reading frames (ORF) located in a single strand of DNA. A single filament therefore acts as a template for the transcription of early (E, Early) and late (L, Late) proteins. The early regions of the viral genome are expressed in cells with non-productive infection and transformed cells, while late regions, including capsidic proteins L1 and L2, are expressed only in cells in which a productive infection occurs. This reflects on an essential characteristic of the virus, that is, on its ability to give a productive infection exclusively in squamous

cells in terminal differentiation and therefore on the fact that the life cycle of the virus is closely correlated with the differentiation program of the squamous epithelium.

Papillomaviruses are characterized by a particular tropism for squamous epithelial cells and the infection usually begins in the basal and parabasal cells of the epithelium, which have a marked proliferative activity. To give infection, the virus must be able to access the "germinative" compartment of the epithelium and the presence of continuous solutions of the skin and mucous membranes is required. In the initial phase of infection, that is, when the virus colonizes the basal and parabasal cells of the epithelium, the viral genome undergoes episomial replication, being present as an extrachromosomal fragment of circular DNA. During productive infection, the expression of viral genes proceeds sequentially following the differentiation phases of the epithelium, starting from the basal and parabasal cells, where the early genes of the virus are more active, up to the upper layers of the epithelium (intermediate and superficial), in which we witness a significant production of capsid proteins and the formation of the complete virion, or the infecting viral particle (Howley & Douglas, 2007). The HPV genome (fig. 1) contains a main regulatory region, consisting of a long control region (LCR), and a region coding for structural and non-structural viral proteins. Most HPVs have six widely studied and described non-structural genes (E1, E2, E4, E5, E6 and E7) and two ORFs (E3 and E8) whose function is not yet well known. Most viral genes are multifunctional. The E1 protein has a DNA replicase activity. E2 regulates the activation and repression of gene transcription and participates in the regulation of viral DNA replication. This activity is mediated by the binding of the E2 protein to the viral genome control region (LCR). E4 codes for a cytoplasmic phosphoprotein, whose function has not yet been identified. HPV has three main oncogenes: E5, E6 and E7. The protein encoded by E5 performs its oncogenic function by interacting and activating specific receptors for growth factors. The E6 protein, in particular that of the high risk types, induces the ubiquitin-dependent degradation of the p53 tumor suppressor, while the E7 protein binds and inactivates the non-phosphorylated form of another tumor suppressor, the retinoblastoma protein pRb. The structural proteins of HPV are essentially two: L1 and L2. L1 proteins make up the viral capsid, which is

composed of 72 L1 pentamers. Neutralizing immunodominant epitopes are localized on L1. The L2 protein is present in less quantity than L1 and participates with the latter in the formation of the capsid. Viral genes can also be classified according to the order in which they are expressed during the replication cycle, and therefore divided into early (early) and late (late) genes. The genes E1, E2, E4, E5, E6 and E7, which encode proteins involved in the regulation of viral replication and cellular transformation, are expressed early in the epithelial basal layers, while L1 and L2, being genes coding for structural proteins, are expressed almost exclusively in the superficial layers of the epithelia in active replication. In the episomial state of the virus, the expression of the E6 and E7 oncogenes is inhibited by the E2 protein. The integration of the viral genome into the cell's DNA causes an interruption of the region encoding the protein E2 and consequently its inhibitory function on the promoters of the oncogen E6 and E7 is lacking. The site of integration of the viral DNA into the chromosomes of the host cell is random, but the type of integration is clonal: the same in all the cells that make up a certain neoplasm. In the epithelium not infected with the virus, basal cells exit the cell cycle immediately after migrating to the parabasal layer, where they undergo a process that leads to differentiation. A number of changes occur in this layer such as cross-linking of intermediate filaments of keratin, the formation of the stratum corneum and the secretion of lipids, which contribute to the formation of a protective physical barrier against the environment. During HPV infection, however, the expression of E6 and E7 in the cell blocks the cell cycle inhibition systems and, consequently, normal cell differentiation is inhibited (26). The two oncogene proteins, E6 and E7, work together to achieve these effects, and are expressed by high-risk viral types as a bicistronic mRNA (27) under the control of the early viral promoter p97. In the non-oncogenic viral types, E6 and E7 can be expressed on different messengers (28). The oncogenic activity of the HPV defined as high risk is mediated by the viral proteins E6 and E7, which bind and inactivate the oncosuppressants p53 and Rb, respectively, in addition to interacting with many other cellular proteins involved in cell proliferation and signaling and in the intercellular adhesion. The normal function of Rb is to block the cell in one stage of the cell cycle, preventing incorrect or harmful divisions. Thus, when Rb is

defective, some mutated cells can continue to divide undisturbed giving rise to the tumor. E7 can also be associated with other factors involved in cell proliferation such as the histone deacetylase enzyme (29). The viral protein E6 complements the function of E7, preventing the induction of apoptosis by inactivating p53. P53 is a transcription factor that regulates the cell cycle with tumor suppressor function for its ability to preserve the stability of the genome through the prevention of mutations. (fig.2)

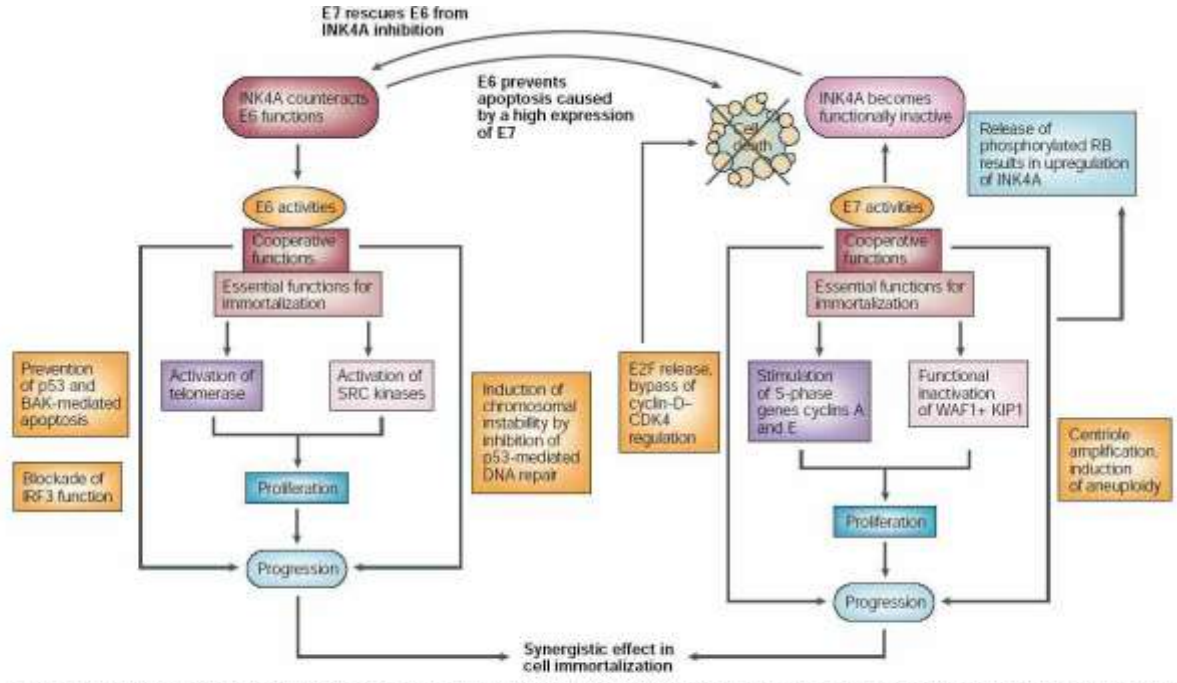

Fig.2: function of E6 and E7 oncoproteins and their interaction with each other in steps that leads to cell immortalizations

L1 e L2

The genes contained in the Late (L) region encode capsid proteins. L1 is the major capsid protein with a molecular weight of 55-60 KDa, while L2 is the minor protein with a weight of about 70 KDa. The capsid consists of 72 capsomers. Each capsomer is a pentamer of 360 L1 protein molecules and 12 to 36 L2 protein molecules. The transcription of these two genes depends on the differentiation state of the keratinocyte. L1 is the most antigenic protein of HPV, it is poorly phosphorylated and can be glycosylated; it is mainly involved in viral DNA binding. L2 mainly plays structural roles, but also various regulatory functions, including binding to secondary receptors; this interaction occurs after

the capsid bond. It participates in the determination of the nuclear localization of the virus and the selective encapsidation of DNA in the viral capsid.

2.2 Classification and genotyping of HPV

There are more than 180 papillomaviruses described (fig.2) and characterized and it is estimated that many more will be identified in future years. They are also very different from each other by guest and by type and site of injury to which they are related. For this reason, the classification of this group of viruses, which includes important human pathogens, took three decades of study and debate. Isolated papillomaviruses are traditionally identified as "types". The classification is based not only on the species from which the viral type was isolated, but above all on the extent and degree of homology between the viral genomes. A viral type is classified by comparing the nucleotide sequence of specific regions of the genome particularly conserved among the various members of the family, such as E6, E7 and L1 ORF.

New sequencing methods and advances in molecular biology (30-31) have made it possible to improve the classification of papillomaviruses. These analyzes also revealed how the genome of these viruses is extremely stable and how 7 recombination or mutation events are so rare that they have a frequency very similar to that of the host organisms of the infection (25). Based on the criteria adopted by the Papillomavirus Nomenclature Committe, to define a new viral type, the sequence homology of its viral genes must be less than 90% compared to those of the known types; higher degrees of homology instead identify a variant of a known type or a subtype thereof (International Papillomavirus Workshop, Quebec 1995). The new HPV is assigned a number, which is established only after the genome has been isolated and fully characterized; then the genome is deposited at the Reference Center for Papillomaviruses (Heidelberg). Based on the alignment of the highly conserved L1 ORF sequence, therefore, HPV viruses are classified into 12 genera, indicated with the first twelve letters of the Greek alphabet. All members belonging to the same gender have a sequence homology of at least 60%. HPV of the same genus with a homology greater than 60-70% are grouped in species,

those with homology between 70 and 90% are grouped in types, those with more than 90% homology in subtypes, and those with homology greater than 98% are considered variants. Five of the twelve genera, alpha, beta, gama, mu and nu are human papillomaviruses while the other seven produce infection in animals. The different viral subgroups correspond to different clinical-pathological characteristics. HPV belonging to the alpha genus are of greater medical importance as they cause genital and mucous membrane cancers. The HPVs of the beta, gamma, mu and nu genus have a skin and mucous tropism, causing vulgar warts, oral and skin lesions and verruciform epidermodysplasia.

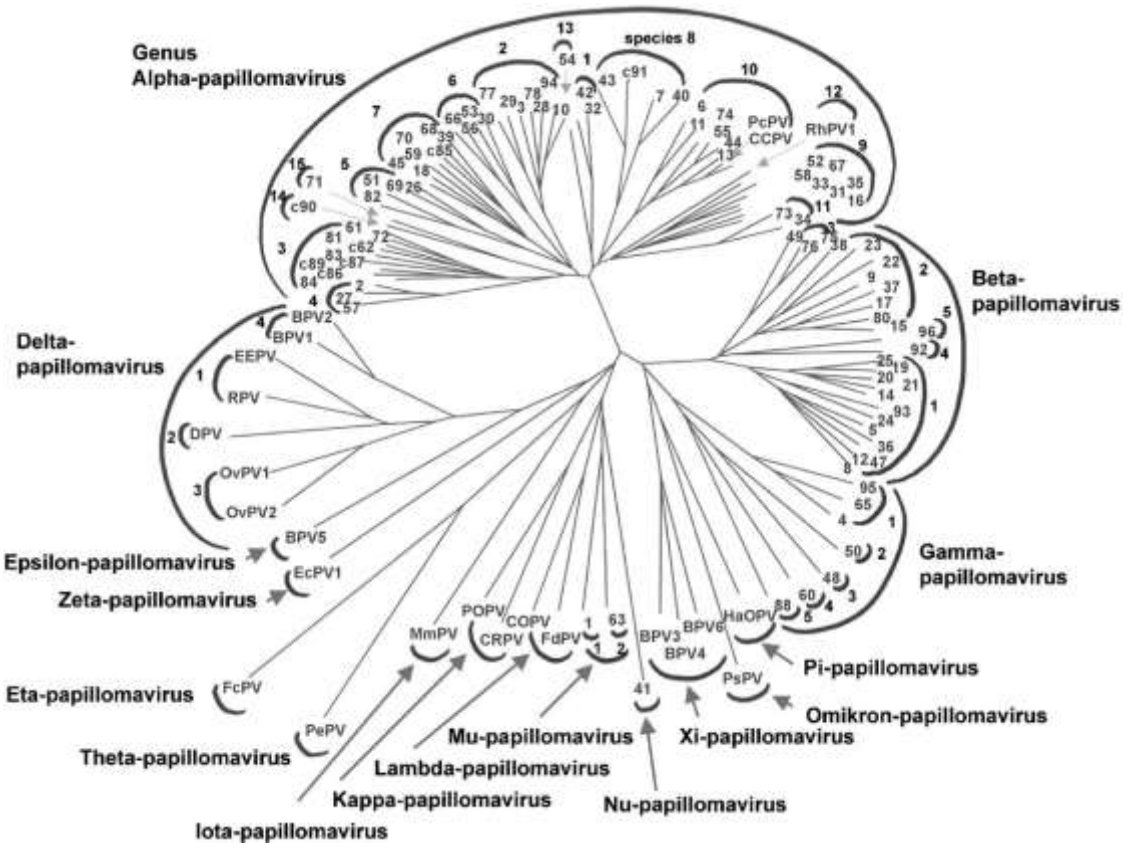

Fig.3. Classification and phylogenetic tree of HPV constructed by comparing the ORF L1 sequence (from Hoory et al., 2008). The purpose of the genotypic classification is to establish correlations between the innumerable viral types, to create a common language that allows to distinguish genus and species (de Villiers, 2004), but above all to investigate the relationships between the taxonomic classifications and the biological and pathological properties of the virus.

In vitro studies have shown that some HPVs with tropism for the mucous membranes and correlated with each other phylogenetically have a common oncogenic potential: the E6 and E7 proteins of this

group of viruses interfere with the cell cycle interacting respectively with the cellular proteins p53 and Rb. From epidemiological studies, however, it has been seen that, within this group of viruses, some have a greater oncogenic potential and are more frequently associated with cancer, others are instead equipped with lower oncogene capabilities; this figure is probably due to a different competence of the E6 and E7 proteins in interfering with cellular proteins. (Tab.1)

Alpha-papillomavirus		
IARC group	Type of HPV	
1	16	The most potent type of HPV as an agent carcinogenic, recognized as a cause of carcinoma in several sites
1	18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59	Sufficient evidence of causal association with cervical carcinoma
2°	68	Limited and strong evidence in humans mechanistic evidence of causal association with cervical cancer
2B	26, 53, 66, 67, 70, 73, 82	Limited evidence in humans of causal association with carcinoma cervical
2B	30, 34, 69, 85, 97	Classified with possible carcinogens by phylogenetic analogy with HPV types with sufficient or limited evidence of carcinogenicity in humans
3	6, 11	Inadequate epidemiological evidence e absence of carcinogenic potential in mechanistic studies
Beta-papillomavirus		
2B	5, 8	Limited evidence of causal association with IV skin cancer
3	Other types of the beta and gamma genres	Inadequate epidemiological evidence and absence of carcinogenic potential in studies mechanistic

Tab. I:

IARC classification of HPV in groups according to oncogenic power (Bouvard et al., 2009).

From the point of view of oncogene capacities we can distinguish high risk HPV, including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, HPV with possible high risk , including types 26, 53 and 66, 68, 73, 82 and low-risk HPVs, including types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 , 81, CP6108. The high risk viral types, also from the phylogenetic point of view, are close to each other, while they are phylogenetically distant from the low risk types (25).

For some HPVs the oncogenic risk is still not well clarified, but phylogenetic studies can give an address in this sense. For example, HPV-67 was initially isolated from intraepithelial vaginal neoplasms (VIN) and it was subsequently ascertained that it is phylogenetically associated with HPV-16, one of the most common high-risk HPV (32). The International Agency for Research on Cancer (IARC), the body of the World Health Organization (WHO) which is responsible for coordinating and conducting research on cancer, has recently classified the main types of HPV based on oncogenic capacity, as summarized in tab. I (33-34). In this classification, viral types are divided into certain carcinogens (group 1), probable carcinogens for humans (group 2A), possible carcinogens for humans (group 2B) and not classifiable as carcinogens for humans. (group 3).

In a recent review of the literature (34), this classification and its impact on decisions regarding choices in screening tests and vaccine development are discussed. The new IARC classification assigns a role of true carcinogen only to a persistent HPV-16 infection; it also defines the importance of HPV-18, especially in relation to adenocarcinoma; includes six alpha-7 (HPV-45) and alpha-9 (HPV-31, HPV-33, HPV-35, HPV-52, HPV-58) types among the eight most important types globally, despite some regional variability in percentage of carcinomas due to each type; establishes a small increase in the etiological contribution of another group of carcinogenic types alpha-5 (HPV-51), alpha-6 (HPV-56), and alpha-7 (HPV-39 and HPV-59) which cause a more o less small percentage of cervical cancer cases in the world. Highlights the fact that the issue regarding some probable or possible carcinogenic viral types such as alpha-7 HPV-68 and alpha-11 HPV-73 remains unresolved

due to the limited data available). The increase in data on HPV-66 allows to re-evaluate this viral type, despite the evidence is still considered limited; the available data show that the finding in HPV-66 cancer is very rare while it is common in women with negative cytology. HPV-53, alpha-6 species, shows the same prevalence in the common population and in extremely rare cases it is associated with cancer. The IARC working group emphasizes, in particular, that the inclusion of HPV-53 among carcinogens in the screening panels can reduce the specificity and positive predictive value of the test without increasing its sensitivity and negative predictive value.

There are many types for which there is no isolated or no evidence of carcinogenicity. For some types there are anecdotal but very interesting cases that deserve further study. For example, there are some reports of individual alpha-9 HPV-70 infections, and few cases of HPV-67 in cancer (35-36). The latter is the only known type of the alpha-9 species not considered as a carcinogen. The IARC classification does not emphasize the possible role of immunosuppression in HPV carcinogenicity (34). To cause cancer, an HPV infection must persist and it is possible that some types of HPV are only weak carcinogens because have poor persistence. For example, the carcinogenicity of the alpha-5 HPV-26 type has been supported by a recent case of multiple periungual cancer in an immunocompromised subject, having a high viral load and an active transcription (37). HPV-26 is a rare type and immunosuppression is probably an important contribution to carcinogenesis. This IARC reclassification is fundamental and must be taken into consideration for all decisions regarding screening tests and vaccine development, as a starting point for future molecular and epidemiological studies.

2.3 HPV Viral Cicle

Infection begins with the access of viral particles inside the cells of the basal layer; for some types of HPV a continuity solution of the stratified epithelium is thought to be necessary. This lesion may not be macroscopically visible and can occur when the skin is exposed to water, when it is abraded or when it is subjected to other environmental conditions that favour the development of microtrauma.

It has been suggested that the virus must infect a stem epithelial cell to maintain the lesion over time (38).

There is strong controversy over the nature of the surface cell receptors that allow the initial adhesion of the virus to the host cell, although several studies have shown a certain dependence on the presence of heparin sulfate (39). Recent work has suggested that the internalization of viral particles is a slow process, and that it can occur through the endocytosis of clathrin-coated vesicles (30); other authors argue that integrin a4þ6 is responsible for the virus to enter the infected cell.

Scapsidation is facilitated by the breaking of intracapsidic disulfide bonds, allowing viral DNA to be transported to the nucleus by nuclear localization signals, although the processes of nuclear scapsidation and nuclear import of the viral genome remain largely unknown. (fig.4)

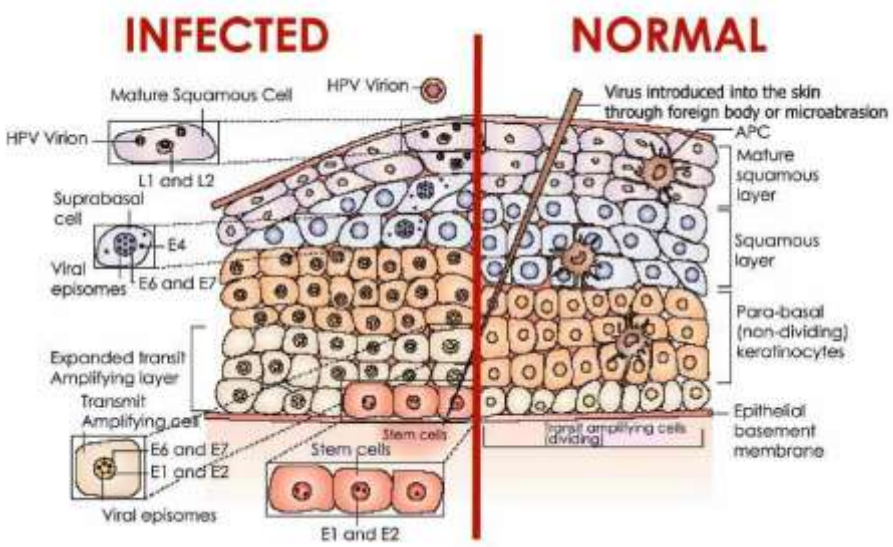

Fig. 4. HPV viral cicle

2.4 Maintenance of genome

The Papillomavirus maintains its genome in episomal form in the basal cells of the epithelium, which constitute the only layer in active division and replication. The HPV infection of these cells leads to the cascade activation of the expression of viral genes that result in the production of extrachromosomal copies (from 20 to 100 copies per cell) of viral DNA (40). The expression of the

gene pattern of these cells is not yet well defined, but targeted research states that the viral E1 / E2 proteins are expressed in order to maintain the HPV DNA in episomal form and to verify the correctness of gene segregation during cell division. The expression of E1 and, probably, of E2 seems to be sufficient for the basal maintenance of viral episomes.

2.4.1. Proliferative phase

In the normal epithelium, the basal cells exit the cell cycle immediately after having migrated to the above basal layer, undergoing the process of terminal differentiation; during this process there are changes in the structure of the intermediate keratin filaments and the secretion of lipids, factors that allow the surface epithelium to create a physical barrier against the surrounding environment. (41)

During HPV infection, the E7 protein (and perhaps also E6) is expressed in these cells, the block to cell cycle progression is abolished and the process of normal terminal differentiation is delayed.

E6 / E7 both have some functions that stimulate the progression of the cell cycle and are associated with some regulators of this; E6 and E7 work in synergy to achieve these effects and, in lesions caused by high-risk HPV such as HPV 16, the two proteins are produced from a bicistronicity mRNA, expressed by the early viral promoter.

2.4.2 Gene amplification

For production of virions, HPV must amplify its genome and insert it within the infecting particles. The late promoter is localized within the E7 open reading frame (ORF) and its upregulation is thought to lead to an increased expression of proteins involved in viral DNA replication, however without directly affecting the expression of proteins E6 and E7, necessary for the entry into the S-phrase of the cell cycle.

The amplification of the viral genome begins in a small group of cells of the proliferative compartment and requires the expression of all early genes, including E4 (41-43) and E5 whose role in replication is not yet fully understood.

The binding of E2 to the upstream regulatory region of the Papillomavirus is necessary for the replication of viral DNA, and the formation of the E1 / E2 complex can allow the replication itself to proceed even in the absence of cellular DNA synthesis.

The molecular mechanism that leads to the activation of the late promoter and to the over-expression of the E1 / E2 complex is not well known; it seems that this promoter is even constitutively expressed in all stages of the viral cycle.

Recent studies suggest that a modest increase in the activity of the promoter during differentiation can increase the levels of E1 and E2 (probably also E4 and E5), consequently leading to an increase in gene copies. The newly replicated genome will serve as a model for the further expression of E1 and E2, an expression that will facilitate an additional amplification of the viral genome.

2.4.3 Assembly of viral particells

HPV encodes two structural proteins, expressed in the most superficial layers of the infected tissue when the amplification of the viral genome has been completed.

The HPV particles consist of a genome of about 8000 bp, placed inside a capsid that contains 360 copies of the major protein L1 and 12 to 36 copies of the minor protein L2. The latter accumulates around nuclear structures, known as "PML bodies", during the assembly of the virus, through the association with a particular transcription factor (43); it is thought that the "PML bodies" are the replication sites of viral DNA and that the L1 / L2 proteins are recalled around them to facilitate the insertion of DNA into the capsid (packaging). Furthermore, the L2 protein improves the efficiency of the "packaging" and increases viral infectivity. Finally, the virus must leave the infected cells and survive in the extra-cellular environment for a variable period of time, before causing a new infection.

2.5 Guest defense mechanisms

The immune system is important in controlling HPV infections. This agrees with the fact that, in immunosuppressed women, SIL (“squamous lesion intraepithelial ") has a greater incidence and

persistence. In particular it is observed the intervention of the cellular and humoral immune system. HPV infections are similar to those mediated by non-lytic viruses, as they do not cause cell destruction, but they are released from infected cells by desquamation. The ideal defense towards this type of infection is a combination of neutralizing antibodies and cell lysis mediated by cytotoxic T lymphocytes (CTL). CTLs act on keratinocytes present in middle layers of the squamous epithelia, where transcription and replication of the virus and where early proteins are abundantly expressed. Despite the proteins late L1 and L2 are not targets of CTLs, as they are expressed in the strata superficial, neutralizing antibodies are directed precisely towards these proteins, however prevent new infections (44). Antibody-mediated neutralization particularly involves serum and IgG Secretory IgA, directed towards virus surface antigens, in order to block them entry into the epithelial mucous membranes. Circulating antibodies and complement can opsonize and agglutinate the viral particles, facilitating the phagocytosis mediated by C3b and Fc receptors. It is unclear whether the antibody response is sufficient to protect them women from infection and whether this protection is lasting. The persistence of warts, even in immunocompetent individuals, it indicates that the immune system is having difficulty in triggering an effective response, due to local or lack of immunosuppression recognition of viral proteins**.**

Antibodies are not always able to eliminate the virus, especially when it enters a latency state, in which the viral DNA is integrated into the cellular DNA. At this point, the intervention of the cellular immunity system is particularly important, mediated by CD8 + cytotoxic T lymphocytes (CTL) and "helper" type T lymphocytes1 (Th1) CD4 +. This system implements intercellular and intracellular surveillance strategies, which prevent the accumulation of cells cancer, both by abolishing the expression of viral oncogenes, and by eliminating the infected cells through apoptosis.

Intracellular regulatory mechanisms prevent the immortalization of cells infected with high-risk HPV. The molecular mechanisms underlying this process involve the modification of viral oncoproteins, through phosphorylation or dephosphorylation. Oncoprotein E7, for example, is phosphorylated by casein kinase II. Another inhibition mechanism is mediated by the interaction between viral

oncoproteins and host proteins, such as inhibitors of cyclin kinases (p21 and p27), which are usually inactivated by E7, but whose overexpression leads to opposite results, blocking E7. Intercellular regulation, on the other hand, prevents the conversion of immortalized cells (which clinically equate to low-grade epithelial lesions) from HPV to a malignant phenotype. This mechanism is mainly mediated by the action of cytokines secreted by Th1 and Th2. Specific cytokines mediate transcription repression of viral oncogenes, unlike IL-6 and IL-17, which enhance the tumorigenicity of cervical cancer cells in nude mice.

TGF-β is one of the cytokines that block the transcription of early HPV genes in immortalized cells, while transcriptional activity remains unchanged in malignant cells. Resistance to TGF-β inhibition is a late event in the course of the development of cervical cancer. Other cytokines that block HPV transcription in immortalized cells are IL-1 and TNF-α.

Studies have shown that TNF-α, secreted by macrophages, also works by repressing the transcription of HPV genes in immortalized cells, but not in malignant cells. This is mainly due to modifications of the transcriptional complex AP-1. The expression of AP-1 in the course of cell differentiation and determines the expression of E6 and E7 in stratified epithelia. While in immortalized cells the AP-1 transcriptional complex at the level of the HPV promoter consists of c-jun / c-jun homodimers, treatment with TNF-α instead leads to the formation of cjun / fra heterodimers (the latter is also activated from retinoic acid, another potent inhibitor of HPV), capable of suppressing the transcription of HPV. In malignant cells, the AP-1 complex is instead formed by c-jun / c-fos heterodimers, which play an important role in the conversion of immortalized cells towards a malignant phenotype.(45). Other important inhibitory cytokines are interferons and IL-2, produced by activated Th1 cells. IL-2 performs an indirect action, mediating the activation of CTL precursors. IL-2 and IFN-α activate NK cells, important in the first days of infection until a specific response mediated by CTL develops in 3-4 days: they destroy infected cells, eliminating potential sources of infection. Interferons, especially β and γ (α is more specific for some cell lines), act both on immortalized cells and on malignant cells, eliminating the virus by inducing an antiviral state in the

cells. The tumor may present mechanisms of resistance to interferons, through the action of E6 and E7, which they interact with factors involved in the synthesis of IFN-α, such as p48, thus blocking its transcription.

The demonstration of a selective inhibition, in vivo, mediated by IFN, of the transcription of HPV in immortalized cells, derives from in situ hybridization experiments, following the transplantation of immortalized cells in nude mice and treatment with IFN.

After three days the transcription of E6 and E7 is reduced more than what happens in vitro. If the same treatment is carried out on malignant cells, the same results are not obtained. These data also agree with clinical observations, in which it is seen that, in low-grade lesions, the expression of E6 and E7 is considerably lower than in high-grade lesions (46).

From these data emerges an important role of cytokine-mediated intercellular HPV control, which is lost with malignant tumor progression (47)

2.5.1 Mechanisms of evasion from the immune system

HPVs, particularly the high-risk subtypes, have evolved a number of mechanisms to evade the defense systems of the target cell, which underlie the progression of tumors in which HPV is involved:

- lead to a decrease in the cellular expression of the major histocompatibility complex of class I (MHCI) and of the transport protein TAP-1, with consequent alterations in the presentation of the antigen. (48)

- they modify the expression of chemokines and cytokines, blocking the communication processes between HPV-infected keratinocytes and the effectors of the immune system; (49)

- guide the cell to escape from apoptotic stimuli mediated by the CD95 / TNF, TRAIL / TNF-α complexes and their related signal transmission mechanisms (50)

The interaction between HPV and the immune system differs from that of other viruses. In fact, while pathogens such as cytomegalovirus produce specific antigens that interfere with the mechanisms of antigen processing, no HPV protein performs this function as a primary purpose. It is the concomitance of several factors a minimize the presentation of the virus to the immune system.

Various alterations of the antigen presentation system, involving proteasome transport mechanisms, HLA receptors and cellular antigen recognition systems, occur, for example, in high grade squamous intraepithelial lesions and carcinoma in situ. This set of mechanisms of evasion from the immune system is crucial for the progression of infections to malignancy.

Another mechanism involves antigen presenting cells. In fact, to be recognized by CTLs, viral antigens must be released from the infected cell and presented by antigen-presenting cells (APC), among which important are Langerhans cells. Following the infection, a decrease and morphological alteration of these cells is observed, a phenomenon that could explain the long persistence of the virus in the cells. The cause could be a decrease in TNF-α, which is a powerful activator of Langerhans cells, or the expression of oncoprotein E7, which interferes with their differentiation. Furthermore, since the HPV infection does not include a viraemic phase, the possibility of an effective presentation decreases antigenic by APCs.

Evasion from apoptotic mechanisms is also particularly important in the course of viral pathogenesis. By definition, apoptosis is a genetically determined program that leads to the activation of caspase-activated deoxyribonucleases. As a result, not only the high molecular weight DNA is cleaved, but also the viral genome that has not yet been encapsulated. Therefore, it is an important process in limiting the spread of viral progeny in the body and in maintaining an adequate ratio between the number of lost and acquired cells; the alteration of this balance can favor tumor progression. The oncogenes of some HPV types interfere with the cell's ability to undergo programmed cell death, as demonstrated using the "TUNEL" technique (which measures the level of DNA fragmentation directly on sections of primary tissue).

HPVs have developed efficient strategies to modulate the response apoptotic following infection. Human papillomavirus E5 protein reduces the amount of the CD95 receptor on the cell surface. However, since E5 is not expressed in cancer cells, the physiological relevance of the decrease in CD95 expression is probably limited to the natural life cycle, in which the temporary block of apoptosis protects the replication intermediates and immature virions from cleavage mediated by

caspases. This data is supported by the discovery that the surface expression of CD95 / TNF receptor I is not quantitatively altered in cells containing immortalized HPV compared to keratinocytes (51) Instead, the E6 / E7 oncoproteins of HPV-16 and HPV-18 efficiently modulate other aspects of apoptotic mechanisms. For example, E6 of HPV-16 sensitizes mammary epithelial cells immortalized in the presence of tamoxifen and other agents that damage DNA to apoptosis, but protects the fibrous cells during natural differentiation in a manner dependent or not on p53. In contrast, human fibroblasts immortalized by E7 are resistant to cell death mediated by TNFα / cycloheximide but not that mediated by TRAIL in keratinocytes (52). Both oncogenes lead to increased apoptosis in human epithelial cells following exposure to hypoxic conditions, (53) while HPV-16 positive cervical cancer cells are less susceptible to CD95-mediated apoptosis(49)

The main effect of E6 on apoptotic mechanisms is the proteolytic inactivation of some pro-apoptotic factors such as p53, Bak, Bax or c-Myc, through the proteasome system, according to the methods previously described. In particular, the inactivation of p53 is a key point in the regulation of apoptosis induced by CD95. The application of CD95-L alone induces cell death in cells immortalized by E7, while keratinocytes expressing E6 or E6 / E7 are resistant (54). The latter can be sensitized to the apoptosis induced by CD95-L following the block of the degradation mediated by the proteasome, preceded by the re-expression of p53 and c-Myc. This observation agrees with the idea that the activation of the CD95 receptor requires additional modifications by p53 in its phosphorylated form. E6 performs its antiapoptotic function also at other levels, in particular by acting on TNF-α, according to a mechanism mediated by p53. TNF-α, unlike CD95, induces other protective mechanisms for the cell, such as those mediated by NF-κB or MAP kinases. E6 prevents the TNF R1 death domain from interacting with FADD. In fact, the C-terminal portion of the receptor is blocked, thus preventing the activation of procaspases 8 and 3, both necessary for efficient transmission of the apoptotic signal E7, as previously described, acts by inactivating Rb, which performs an anti-apoptotic action; therefore, the decrease in intracellular levels of Rb, caused by E7, favors the triggering of apoptotic mechanisms (55)

When E7 is introduced into human fibroblasts it leads to decreased procaspase 8 which protects against TNF-α / CHX-induced cell death. E7 also plays an important role in modulating the balance between histone deacetylase (HDAC) and histone acetylase (HAT), as it binds HDAC through the Mi2β protein; E6 also affects this equilibrium, abrogating the costimulatory function of CBP and p300. It has been shown that HDAC inhibition induces growth arrest and apoptosis in cells expressing E7, but not when only the E6 protein is expressed (56).

Chapter 3

HPV DIAGNOSIS

Chapter 3

Diagnosis of HPV infection

HPV cannot be grown, therefore the methods for detecting the presence of the virus are based exclusively on the search for nucleic acids.

A good method, in addition to being specific and sensitive, should be able to distinguish the viral genotype and identify the largest number of viral types. Not all the tests currently available meet these characteristics: some allow to identify only the most frequent viral genotypes and exclude some rarer ones, considered less relevant for screening programs; others allow to distinguish only between high and low risk HPV. The method must also allow to identify co-infections from multiple types of HPV, since 20-30% of HPV infections are due to multiple viral types and it is important to determine the contribution of each virus in the infection.

Several in-house tests and protocols have been developed over the past two decades and applied to randomized trials and clinical trials; some laboratories still use them even for clinical practice and may be indispensable in some particular situations. These tests are essentially based on thermite PCR amplification using consensus primer and sequencing. However, most diagnostic laboratories use one of the many commercial tests now available for the HPV test for the routine. There are at least 125 commercially available tests and 84 variants of some of these that allow you to identify a panel of viral types larger than the original test. A recent review (57) has divided the HPV tests on the market into six different groups and related subgroups: hrHPV DNA test, hrHPV DNA test which allow partial genotyping of the main types of high-risk HPV at the same time or deferred, HPV DNA tests that allow the genotyping of all types of HPV, HPV DNA type- or group-specific genotyping HPV type- or genotyping tests-specific group, tests that evaluate the hrHPV mRNA E6 / E7, HPV tests based on in situ hybridization.

In the application of the HPV test to secondary screening, that is, in cases with cytological alterations, tests are often used that allow a wide

genotyping and evaluation of multiple infections. Most of these tests on the market are based on end-point PCR method, with genotype detection through different techniques such as hybridization on reverse phase strip (Inno-LiPA, Linear Array) or microarray (Papillocheck), allowing the detection of genotypes with low and intermediate oncogenic risk.

3.1. Polymerase chain reaction (PCR) with consensus

Primer PCR is the most used method for identifying HPV DNA, and there are two possible approaches: using a type-specific PCR or a broad-spectrum PCR. Type-specific primers allow to detect the presence of a single viral type per reaction. In this case, the design of the primers is very important which, if they map in a too conserved region, could also interact with other viral types. This approach is therefore useful if a specific viral type is investigated or suspected (eg HPV-16 in a tumor of the uterine cervix), but if a complete genotypic investigation is required, this approach would be investigative, having to set up separate reactions for each type viral (58). The use of broad-spectrum primers is therefore the most used approach. In this case, the primers target a region of the viral genome highly conserved in the different types of HPV, in order to recognize a wide spectrum of genotypes. The most suitable genomic region in this sense is the L1 region, although primers that map to the E1 gene have been described in the literature (59). Primer systems can be designed in three ways to obtain a broad-spectrum PCR (45). The first uses a pair of primers which maps to a conserved region, but which is fully complementary to one or a few genotypes. In order for more genotypes to be recognized, the reaction must be conducted at a low annealing temperature. An example of such an approach is the GP5 + / GP6 + system which amplifies an L1 region of 150 pb (annealing temperature of 40 ° C) (35).

The second approach uses primers that contain one or more degenerate bases to compensate for the sequence differences between the different viral types. Such primers do not require low annealing temperatures (60). Examples of degenerated primers are the MY09 / MY11 system and the CPI / II system, which include a complex mixture of primers containing degenerated bases (different oligonucleotides) to compensate for the sequence differences between the different viral types. The

first system amplifies an L1 region of 450 pb (annealing temperature of 54 ° C), the second system instead amplifies an E1 ORF region of 188 pb. The disadvantage of these systems is that the degenerations are not highly reproducible and therefore present variations between one batch of primer and the other, and above all differences in the amplification effectiveness of each genotype. The PGMY system was developed precisely to increase the reproducibility and sensitivity of the MY09 / MY11 system, using a pool of oligonucleotides that map in the same region of L1 ORF. The viral types were grouped based on the sequence homology of each of the binding regions of the two primers; on the basis of these groupings, a set of 5 oligonucleotides that make up the PGMY11 primer pool and a set of 13 oligonucleotides that make up the PGMY09 pool were designed (42). The third method combines a number of distinct primers that map into the same genomic region. They do not contain degenerate sites, but inosine which has the ability to pair with all the nucleotides present in the points where there are sequence differences. This primer mixture has the advantage that oligonucleotides are synthesized in a highly reproducible way and that PCR can be conducted at optimal temperatures. Examples of these primer sets published in the literature are the SPF10 system, which amplifies a small 65-pb fragment of the L1 ORF of 43 viral types (10) and the Amplicor system, which amplifies a sequence of 170 pb always in L1 ("high risk" genotypes), using 5 forward primers and 7 reverse (33). The choice of the size of the amplification product is also important, because the efficiency of the reaction increases the smaller the amplified. Each set of primers also allows you to amplify the different viral types with different efficiency. For example CPI / II primers detect viral types -1, -2, -3, -4, -5, -6b, -7, -8, -9, -10a, -11, -12, -14a, -16, -17, -18, -19, -20, -21, -22, -24, -25, -31, -33, -36, -37, -38, -39 and -46, while the -15, -23, -49 and -50 are poorly amplified and the -41 is not amplified (61). The association of two PCRs with different primers such as CPI / II and MY09 / 11 or GP5 + / 6 + and MY09 / 11, allows to increase the sensitivity of the test. Sensitivity can also be increased with a nested PCR method. For example it is possible to perform a PCR with primer MY09 / 11 followed by a PCR with primer GP5 + / 6 +, which flank a sequence internal to the first amplified.

3.2. Commercial tests for the diagnosis of hpv hrHPV DNA test

This category includes qualitative or semi-quantitative tests, which allow to identify types of HPV considered oncogenic in aggregate, using different technologies, without distinguishing the single type or the different types of HPV present in the sample. This group includes two tests, both FDA approved and commonly used for the triage of women with dubious cytology, in the post-treatment follow-up of high-grade intraepithelial neoplasms (CIN) and in primary screening: Hybrid Capture 2 (HC2) HPV DNA test (HC2, Digene Corp., USA), labelled Qiagen (MD, USA) and Cervista HPV HR Test (Hologic, Madison, WI).

The HC2 system is based on a signal amplification method that uses two different mixtures of RNA probes: the first contains the probes for the high types (16, 18, 31,33,35, 39, 45, 51, 52 , 56, 58, 59, 68), the second those at low risk (6, 11, 42, 43, 44). The denatured DNA is hybridized with the RNA probes present in the mixture. The possibly formed DNA-RNA hybrids are captured in solid phase in suitable wells and reacted with monoclonal antibodies labelled with peroxidase; then the signal is detected by chemiluminescence, obtaining a semiquantitative measurement. The analytical sensitivity of the method is 1 pg / ml of DNA which corresponds to 105 genomic copies. This method has some limitations related to the lower sensitivity compared to the PCR methods, it presents risks of cross reactivity between the two cocktail of probes and above all it distinguishes high and low risk genotypes, but does not allow genotyping (12). On the other hand, this test has been widely validated in the clinical field in screening programs, demonstrating its effectiveness in the prevention of cervical cancer and pre-invasive cervical lesions. HC2 is currently the most widely used test, and guidelines recommend that any new test, to be usable in a clinical trial for cervical cancer, should have clinical characteristics at least equivalent to HC2 (40).

The Cervista HPV HR test is based on hybridization and signal amplification with Invader chemistry and identifies 14 types of high-risk HPV through a fully automated method.

hrHPV DNA tests that allow partial genotyping of the main types of high-risk HPV at the same time or on a delayed basis.

This group includes some new tests that allow to detect 13 or 14 high-risk viral types and simultaneously or in a separate reaction, to evaluate the presence of HPV16 and HPV18. These tests were developed on the basis of the results of clinical studies, which show that these two viral types have a much higher oncogenic potential than the other genotypes.(63)

This category includes the cobas 4800 HPV test (Roche Molecular Systems Inc., Almeda, CA, USA). The test, approved by FDA, allows you to screen for 12 high-risk genotypes and to individually evaluate the presence of HPV16 and 18. The Cervista HPV16 / 18 (Hologic) test, approved by FDA, is used in cases of positivity to the Cervista HPV test HR and is therefore included in the group of tests performed "deferred".

To these two FDA approved tests, is added the clinically validated Abbott RealTime High Risk HPV test (Abbott Molecular, Des Plaines, IL). The test allows simultaneous detection of high-risk HPV 14 and HPV16 and HPV18 using real-time PCR 14.(64)

Tests that allow the genotyping of all types of HPV

Most of the tests included in this category use the reverse hybridization method for genotyping a broad spectrum of HPV types. A viral genome sequence is first amplified by PCR, the amplicon obtained is denatured and detected by hybridization with specific probes immobilized on strips, filters or microplates and a colorimetric or chemiluminescence reaction.(65)

The tests in this group for which there is more data in the literature are the Linear Array HPV Genotyping Test (Roche Molecular Systems Inc., Alameda, CA, USA), the HPV SPF10 LiPA25 (Labo Bio-Medical Products, Ev Rijswijk, Nederlands) and several versions of the INNO-LiPA HPV Genotyping test (Innogenetics NV, Gent, Belgium).

The principle of reverse hybridization has also been used for the development of microarray-based tests. In this case, type-specific probes can be immobilized on microarray or micro chip slides, allowing to simultaneously distinguish more than 100 types of HPV (36). The tests of this type on

which there is more data in the literature are the PapilloCheck HPV-screening Test / Papillocheck High-risk Test (greiner Bio-One, Frickenhausen, Germany) and the Clart HPV2-Papillomavirus Clinical Arrays (Gnomica, Coslada, Spain).

Oligonucleotide probes can be fixed on even very different surfaces, as in the case of the papillomavirus genotyping (MPG) multiplex system, recently described, in which the PCR product obtained with the GP5 + / GP6 + primers is detected by means of fluorescent probes linked to beads of polystyrene (luminex suspension array technology). The pearls are colored with different combinations of two distinct fluorophores and it is possible to create up to 100 different types of pearls absorb at different spectra and on each type it is possible to attach a different type of probe. This method would therefore allow to distinguish more than 100 viral types at the same time (50)

HPV type- or group-specific genotyping test

As mentioned before, type-specific primers allow to detect the presence of a single viral type per reaction. This approach is therefore useful when a specific viral type is investigated or suspected (eg HPV-16 in cervical cancer).

Specific type primers are used above all by real-time PCR, which has the advantage of allowing a quantification of the viral load (52). The usefulness of quantifying the load of the viral type identified has been suggested by some authors for HPV-16-positive cervical tumors. A high burden of HPV-16 would appear to be correlated with high-grade CIN and invasive carcinoma or with an increased risk of developing CIN2-3 (15). This correlation would not seem demonstrable for the other viral genotypes (34). Real-time PCR can also be used to distinguish the different genotypes or by setting up separate reactions for each viral type (58) or by means of a mixture of probes; the probes often have different hybridization characteristics and the standardization of the reaction is in this case rather complex (61). However, some works have been published in the literature that use multiplex real-time PCR with Beacon probes, in systems that allow at the same time to genotype and quantify viruses (56).

Direct sequencing

Rapid sequencing methods are used for the identification of HPV after amplification with consensus primers in many in-house protocols. These methods allow to identify almost all known genotypes, but they also have important limitations. In fact, sequencing does not allow to identify the genotype when the viral load is low and cannot be used for cases of co-infections from multiple genotypes. The traditional sequencing technique consists in the generation of single-helix fragments by interrupting the extension of the chain. This is done with a mixture containing a complementary primer apart from the nucleotide sequence; the enzyme DNA polymerase; deoxyribonucleotides triphosphate (dATP, dTTP, dCTP, dGTP); a small amount of 2'-3'-dixoxy analogues (ddNTP), which have only one hydrogen atom instead of the 3'-OH group and are labelled with different fluorophores. The primer pairs to the template strand and is extended by the DNA polymerase. This adds dNTPs complementary to the template strand to the free end of the primer, but occasionally randomly adds a ddNTP instead of adding a dNTP. (66) With the incorporation of the ddNTP the synthesis of the complementary strand stops, since the ddNTPs do not allow the formation of the bond with the next nucleotide triphosphate. The dNTPs have a 3'OH in the deoxyribose sugar which allows the addition of nucleotides, the ddNTPs instead have a 3'-H which does not allow such addition. In this way, DNA fragments of various lengths are obtained, which differ in the type of ddNTP with which they end. These fragments are separated by capillary electrophoresis and their migration is detected thanks to the emission of fluorescence caused by a laser. The information is then integrated and transformed into colored peaks (electropherogram) (67).

Once the HPV sequence has been identified, the genotype can be inferred in two ways. The fastest method is to consult a database of sequences with which to search for the homology between the sequence found and the known ones. The best known example of a database on the Internet is BLAST (http://www.ncbi.nlm.nih.gov). The other method instead consists in a phylogenetic analysis in which the sequence found is aligned with a certain number of known sequences, representative of the different genotypes (50). A phylogenetic tree is thus constructed from which it is possible to deduce

the genotype on the basis of the evolutionary relationships between the new sequence and known sequences.

New sequencing methods: pyrosequencing and ultradeep sequencing

Pyrosequencing is a bio luminometric method, alternative to sequencing by electrophoresis (68). It is based on a cascade of coupled enzymatic reactions, which use DNA polymerase ATP sulphorylase and luciferase to monitor DNA synthesis by measuring the pyrophosphate released following the attachment of a dNTP to the polymerized strand. Compared to classic sequencing, this technique has greater accuracy, flexibility and avoids the use of labelled primers and nucleotides and gel electrophoresis. Already in 2001 the method was applied by some authors to the typing of HPV(56) to sequence amplified of 20-40 bp (GP5 + / 6 +). The comparison with classical sequencing gave 100% concordant results. Pyrosequencing allows for fast and efficient genotyping; it also allows to identify new types of HPV, which with hybridization methods are not recognized or cross-react, and to identify sequence variants due to mutations. According to the same authors, the advantages of pyrosequencing are most noticeable when a pool of multiple type-specific primers is used for sequencing (56). With this approach it is possible to: a) detect multiple infections and discriminate the dominant and sub dominant genotypes as a whole; b) identify genotypes of interest in amplifiers containing non-specific amplification products; c) detect species / types / target DNA of interest with sequence specific primers in amplified products obtained with degenerated primers or with multiplex PCR; d) detect low-yield amplifiers whose PCR would normally be repeated.

One pyrosequencing system is the 454 deep sequencing system (Life Sience, Roche), which carries out high-throughput parallel massive DNA sequencing using a novel synthesis sequencing approach and sequencing up to 10Mbp of genome.

INNO-LiPA HPV Genotyping Extra II

Recent studies have provided evidence for a difference in oncogenic potential between the different hr-HPVs, arguing for the importance of HPV genotyping in addition to the “hr-HPV plus/minus”

screening. Outside of the clinical setting, HPV genotyping is a key characteristic of studies evaluating the epidemiology of HPV infections worldwide. Although a number of HPV genotyping assays have been used in such studies, a reliable comparison between the diagnostic and epidemiological data generated is difficult, since data on the intertest comparisons between the different genotyping assays are limited. The SPF10-INNO LiPA assay is capable of amplifying up to 43 different genotypes and providing type-specific genotype information for 25 different HPV genotypes simultaneously, has been extensively tested, and has proven to be highly sensitive and specific.The Roche Linear Array (LA) HPV genotyping test (Roche Molecular Systems, Inc., Branchburg, NJ) is a recently launched new HPV genotyping assay able to genotype 37 HPV types, concurrently assessing human _-globin. The full spectrum of HPV genotypes amplified by the PGMY primer system used in the Roche Linear Array HPV genotyping test has not been assessed beyond the 37 genotypes probed. In essence, both assays could be used for genotyping analysis.

The INNO-LiPA is a line probe assay based on the principle of reverse hybridization for qualitative detection and identification of 32 different HPV types, including 13 hrHPV (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59 and HPV68), 6 possible hrHPV (HPV26, HPV53, HPV66, HPV70, HPV73 and HPV82), 9 low-risk HPV (HPV6, HPV11, HPV40,HPV42, HPV43, HPV44, HPV54, HPV61 and HPV81) plus 4 other HPV genotypes (HPV62, HPV67,HPV83 and HPV89). INNO-LiPA uses the biotinylated consensus primers (SPF10) to amplify a 65-bp region within the L1 region of multiple alpha HPV types. The resulting biotinylated amplicons are then denatured and hybridized with specific oligonucleotide probes. A primer set for the amplification of the human HLA-DPB1 gene is included to monitor sample quality and extraction. The INNO-LiPA assay (sample incubation, stringent wash and color development) was performed fully automated using the AutoBlot 3000H (Bio-Rad Laboratories Inc., Hercules, CA, USA). Interpretation of the developed strips was done by scanning and automated interpretation using with the LiRAS for LiPA HPV software (Version 3.01, Fujirebio Europe, Ghent, Belgium).

Chapter 4

HPV IN THE HEAD NECK DISTRICT: MOLECULAR SCREENING FOR AN EPIDEMIOLOGICAL EVALUATION ON ITALIAN SAMPLES

PHD project

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Chapter 4

HPV IN THE HEAD NECK DISTRICT: MOLECULAR SCREENING FOR AN EPIDEMIOLOGICAL EVALUATION ON ITALIAN SAMPLES

DESIGN AND PROJECTUALITY.

During the first year of attendance at the Pathology and Oral Medicine Outpatient Clinic of the Cà Granda IRCCS Hospital in Milan, the design bases were laid for a search for HPV serotypes particularly related to the possible predisposition and onset of oral lesion muco-cutaneous and oral and oro-pharyngeal neoplasms.

In order to correctly consider the design bases of the study, the following objectives were considered in data collection:

INTRODUCTION

• The place of research and clinical and epidemiological assessments was the Pathology and Oral Medicine Outpatient Clinic of the Cà Granda IRCCS Foundation Hospital in Milan;

• Laboratory diagnostic reference was considered the U.O.C. of Pathological Anatomy of the Polyclinic Hospital of Milan Fondazione Cà Granda IRCCS;

• Laboratory diagnostic reference was considered the OU. of Virology and Serodiagnosis of the Cà Granda IRCCS Foundation Hospital of Milan;

• Laboratory diagnostic reference was considered the OU. Microbiology of the Cà Granda IRCCS Foundation Polyclinic Hospital of Milan;

• All patients in the Pathology and Oral Medicine Outpatient Clinic of the Cà Granda IRCCS Hospital of Milan, have always been included in the context of an IT and numerical file with indication of the category of oral lesions they belong to;

• Each medical record was constantly updated with personal-anamnestic, clinical and diagnostic-instrumental data;

• Each patient investigated was duly informed of the clinical research;

• Each patient investigated joined the clinical-cognitive research spontaneously;

• The research and clinical material has been collected and kept in a place accessible only to professionals and protected by the Privacy decree.

OBJECTIVES OF THE FIRST KNOWLEDGE PHASE OF THE RESEARCH

Materials and methods:

1st Objective

Research in the field of cases of the Oral Pathology and Medicine Outpatient Clinic, of subjects investigated through biopsy and histopathological examination for HPV pathologies related to the mucous membranes of the oral cavity. Population studied and treated from 2005 to December 2016.

2nd Objective

Research in the case history of the Oral Pathology and Medicine Outpatient Clinic, of subjects investigated through biopsy sampling and histopathological examination for correlated non-HPV pathologies where the presence of koilocytes or the presence of koilocytosis are reported in the context of lesions affecting the oral mucous membranes. Population studied and treated from 2005 to December 2016

3rd Objective

Research in the case history of the Oral Pathology and Medicine Outpatient Clinic, of the total number of subjects investigated through biopsy sampling and histopathological examination for correlated and non-HPV related HPV pathologies where the presence of koilocytes or the presence of

koilocytosis in the context of lesions affecting the oral mucosa. Population studied and treated from 2005 to December 2016

4th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with an age division. Population studied and treated from 2005 to December 2016

5th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with a gender division. Population studied and treated from 2005 to December 2016

6th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with a division following a topographical criterion. Population studied and treated from 2005 to December 2016

7th Objective

Division by type of molecular diagnostic investigation method.

8th Objective

Division by type of serotypes identified.

RESULTS

1st Objective

Research in the field of afferent cases and relating to the Oral Pathology and Medicine Outpatient Clinic, of subjects investigated through biopsy sampling and histopathological examination for HPV

pathologies related to the mucous membranes of the oral cavity. Population studied and treated from January 2005 to December 2016. Clinical activity of 12 calendar years.

In the corresponding period, 2322 biopsies (incisional and excisional) were performed.

From the analysis of the histo-pathological reports of the same U.O. of the Policlinico Hospital of Milan we had histopathological diagnostic confirmations and confirmations of pathologies directly related to HPV of 148 cases. 6.37% diagnosis of correlable HPV lesions.

Specifically:

• oral vulgar warts number 49 2.11%

• squamous papillomas number 81 3.48%

• accuminate warts number 18 0.77%.

All values refer to 12 years of clinical activity.

Therefore:

• 2322 biopsies

• 148 cases of HPV injury

• HPV percentages in biopsied lesions

2nd Objective

Research in the case history of the Oral Pathology and Medicine Outpatient Clinic, of subjects investigated through biopsy sampling and histopathological examination for correlated non-HPV pathologies where the presence of koilocytes or the presence of koilocytosis are reported in the context of lesions affecting the oral mucous membranes. Population studied and treated from 2005 to December 2016

In the corresponding period, 2322 biopsies (incisional and excisional) were performed.

From the analysis of the histo-pathological reports of the same U.O. of the Policlinico Hospital of Milan we had histo-pathological findings with the presence of coilocytes or histomorphological behaviour of koilocytosis in 97 cases. Percentage of 4.17% of non-specific diagnoses of HPV lesions

but of tissues with possible contamination (recent or past HPV) and with signs of contact with the virus.

Specifically:

• Oral leucoplakias and hyperkeratosis n. 23 0.99%

• Oral lichen n 36 1.55%

• Proliferative hairy leucoplakia n. 9 0. 38%

• Erosive and erythematous lichen n. 18 0.77%

• Erosions and traumatic ulcerations number 11 0.47%

Therefore:

• 2322 biopsies

• 97 cases and with a percentage of 4.17% of non-specific diagnoses of HPV lesions but of tissues with koilocytosis.

3rd Objective

Research in the case history of the Oral Pathology and Medicine Outpatient Clinic, of the total number of subjects investigated through biopsy sampling and histopathological examination for correlated and non-HPV related HPV pathologies where the presence of koilocytes or the presence of koilocytosis in the context of lesions affecting the oral mucosa. Population studied and treated from 2005 to December 2016.

• In the corresponding period, 2322 biopsies were performed (incisional and excisional)

• In the corresponding period, we had 148 case reports with histopathologically documented HPV related lesions. Percentage of 6.37%

• In the corresponding period we had 97 reports of cases of non-HPV related pathologies but with the presence of koilocytes or histo-morphological behaviours of koilocytosis. Percentage of 4.17%.

• Total histopathological reports with signs of presence or contagion of HPV number 245, equal to 10.55%.

This is evidence of a wide spread of the virus in subjects where there are related and non-related HPV tissue lesions, but with signs of oral lesions.

4th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with an age division. Population studied and treated from 2005 to December 2016.

• Total of 2322 biopsies

• 148 related HPV lesions. Percentage of 6.37%

• 97 case reports of correlated non-HPV diseases. Percentage of 4.17%.

• 245 reports of the presence or occurrence of HPV infection, equal to 10.55%.

Age groups considered and related to 148 related HPV lesions:

• Age from 0 to 6 years 4 2.70%

• Age from 7 to 12 years 23 18.24%

• Age from 13 to 20 years 45 30.40%

• Age from 21 to 40 years 53 35.81%

• Age from 41 to 60 years. 14 9.45%

• Age from 61 to 90 years 9 6.08%

As evidence that infections occur more in the stages of life where human and social relationships occur most.

Age groups considered and related to 97 reports of cases of correlated non-HPV diseases:

• Age from 0 to 6 years 0 0%

• Age from 7 to 12 years 3 3.09%

• Age from 13 to 20 years 5 5.15 %

• Age from 21 to 40 years 13 16.40%

• Age from 41 to 60 years. 32 32.98%

• Age from 61 to 90 years 44 45.36%

As evidence that infections occur in the ages where there is greater exposure; the lack of lesions with asymptomatic pictures could be interpreted as inactive infections and traceable even after years through a histo-pathological examination for correlated non-HPV lesion.

5th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with a gender division. Population studied and treated from 2005 to December 2016

Breakdown by gender of 245 HPV presence or infection reports.

• Female 126 51.42%

• Male 119 48.57%

As evidence of a uniform spread of the virus and equally distributed in the two sexes.

6th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with a division following a topographical criterion. Population studied and treated from 2005 to December 2016

• 148 related HPV lesions. Percentage of 6.37% of 2322

• 97 case reports of correlated non-HPV diseases. Percentage of 4.17%. of 2322

Topographical criterion which includes:

• anterior regions of the mouth,

including the lips and anterior vestibules

• middle regions of the mouth

at the end of the hard palate and at the anterior region of the lingual V

• posterior regions of the mouth

including oro-pharynx.

Topographic criterion applied to 148 related HPV lesions

• anterior regions of the mouth 75 50.67%

• middle regions of the mouth 59 39.86%

• posterior regions of the mouth 14 9.45%

Topographic criterion applied to 97 unrelated HPV lesions

• anterior regions of the mouth 11 11.34%

• middle regions of the mouth 27 27.83%

• posterior regions of the mouth 59 60.82%

Results testify to the spread of viruses not particularly related to AC. oral and not often linked to diffusion for sexual practices.

The presence of the virus, in the posterior regions of the mouth, on the other hand, testifies to the presence of strains that do not manifest themselves with clinically objectionable pathologies but traceable even after years of permanence and more expression of sexual practices.

Objective n.7

All our biopsies were investigated using this molecular diagnostic method by the U.O. of Virology and Serodiagnosis of the Cà Granda IRCCS Foundation Hospital of Milan.

A viral genome sequence is first amplified by PCR. The amplicon obtained is denatured and detected by hybridization with specific probes immobilized on strips, filters or micro-plates and colorimetric or chemiluminescence reaction. In vitro PCR reconstructs a specific step of cell duplication: the reconstitution (synthesis) of a "complete" (double-stranded) DNA segment starting from a single-stranded strand. The missing strand is reconstructed starting from a series of nucleotides, the elementary "building blocks" that make up the nucleic acids, which are arranged in the correct sequence, complementary to that of the affected DNA.

This process is carried out in nature by enzymes called DNA-polymerases, which are able to progressively synthesize a new DNA strand under the following conditions:

• the nucleotides to be polymerized must be available in the form of deoxy-ribonucleosides-triphosphates (dNTP);

• the DNA must be denatured, ie the two helices that make up the strands must already be separated;

• the segment to be reconstructed can only be prolonged, ie it is not possible to synthesize a new strand starting from zero;

• appropriate conditions of temperature, pH, etc. must also be observed.

It is therefore possible to reconstruct the conditions that lead to the formation of new DNA segments, by putting in solution:

• even a small amount of the segment of DNA to be reproduced;

• an appropriate quantity of free nucleotides to make up the new filaments;

• suitable "primers", called primers, consisting of short DNA sequences (oligonucleotides) complementary at the 5 'and 3' ends of the two filaments of the segment to be reproduced;

• a thermo-resistant DNA polymerase (it does not need to come from the same organism whose DNA is to be replicated);

• a buffer that serves to keep the pH suitable for the reaction stable;

• other supporting elements, such as magnesium ions, essential for the correct functioning of DNA polymerase;

To start the polymerase reaction (phase of extension of the filament starting from the primer 5 ') it is first necessary to provide for the separation of the DNA strands (denaturation phase), then to create the bond between the primers and their complementary regions of the filaments of denatured DNA (annealing phase). However, this process is incompatible with human DNA polymerase, which is destroyed at the temperatures necessary for denaturation (96-99 C).

To overcome this drawback, use is made of polymerases belonging to thermophilic organisms that are not inactivated by high temperatures, for example the Taq polymerase coming from the thermophilic bacterium Thermus aquaticus. This allows to carry out several PCR cycles in sequence, in each of which the DNA synthesized in the previous phases is also duplicated, obtaining a chain reaction that allows an extremely rapid multiplication of the genetic material of interest.

Subsequently, the method called LIPA (linear probe access) uses probes linked to a nitrocellulose membrane that "capture" any specific DNA sequences.

This method is based on the link between biotin and streptavidin and consists of three steps:

1) The biotin-labelled amplified DNA is hybridized with the probe

2) the biotinylated hybrid is incubated with streptavidin linked to the enzyme

3) the substrate is put in contact with the DNA-biotin-streptavidin-enzyme complex and develops a colorimetric reaction.

As part of our research, over the 12-year period, the data refer to laboratory investigations of the U: o: Pathological Anatomy alone, where methods such as:

• Broad spectrum PCR

• type-specific PCR

The use of broad-spectrum primers appears to be the most used approach. In this case the primers target a highly conserved region of the viral genome in the different HPV types, in order to recognize a broad spectrum of genotypes. The most suitable genomic region in this sense is the L1 region, although primers that map to the E1 gene have been described in the literature.

Type-specific primers allow detection of the presence of a single viral type per reaction. This approach is therefore useful if a specific viral type is investigated or suspected eg. HPV-16 in oral cancer.

At our pathological anatomy, broad-spectrum investigations and specific investigations for HPV 16 are carried out at our routine request.

• In the context of the research of this last period, the diagnostic collaboration with Pathological Anatomy has expanded with the U.O. of Virologists Virology and serodiagnosis of the Polyclinic Hospital of Milan Fondazione Cà Granda IRCCS;

• in Virology and serodiagnosis of the Cà Granda IRCCS Foundation Hospital of Milan;

Specific tests have been applied at the Virology facility that allow genotyping of all types of HPV. Most of the tests included in this category use the reverse hybridization method of a broad spectrum of HPV types. A viral genome sequence is first amplified by PCR, the amplicon obtained is denatured and detected by hybridization with specific probes immobilized on strips, filters or micro-plates and colorimetric or chemiluminescence reaction. In this case the LIPA test was used.

Objective n. 8

Analysing the results of studies conducted in various parts of the world, the IARC (International Agency for Research on Cancer) has more recently officially identified types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 as carcinogens, while HPV types 6 and HPV 11 are considered possibly carcinogenic to humans while HPV types 6 and HPV 11 are considered possibly carcinogenic to humans.

Classification based on the Tropism of the virus: they are divided into two broad categories, cutaneous HPV and mucosal HPV. Cutaneous HPVs cause skin lesions such as common or vulgar warts (they are the most common forms), they are frequently localized on the hands and feet; another part of cutaneous HPV type is associated with verruciform epidermodysplasia.

Mucosal HPVs cause different types of lesions affecting the genital tract in both women and men; HPV 6 and HPV 11, belonging to the low-risk types, lead to the formation of acuminate warts (benign warts), while the high-risk types are found in squamous intraepithelial lesions that can progress to invasive squamous carcinoma

Of 148 lesions analysed we notice:

- oral vulgar warts n. 49 HPV genotype 10
- squamous papilloma n. 81 HPV genotype 6
- acuminate warts n. 18 HPV genotype 6

OBJECTIVES OF THE SECOND EXPLORATION PHASE OF THE RESEARCH

Materials and methods

1st Objective

Recruitment of subjects investigated through biopsy and histopathological examination for the diagnosis of HPV pathologies related to the mucous membranes of the oral cavity (squamous papilloma, acute condyloma, verruca vulgaris) . 12-month recruitment period from October 2018 to October 2019.

2nd Objective

Recruitment of patients with hyperkeratotic lesions of the oral cavity, investigated through biopsy sampling and histopathological examination for pathologies and the diagnosis of non-HPV related pathologies, in the context of the Oral Medicine and Pathology Outpatient Clinic. The presence of histological signs of infection (presence of coilocytes or the presence of koilocytosis) was evaluated on the samples. 12-month recruitment period from October 2018 to October 2019.

3rd Objective

Collection and classification of clinical-anamnestic data and the outcomes of histopathological and molecular examinations in the context of the caseloads of all subjects recruited in the Oral Medicine and Pathology Outpatient Clinic investigated through biopsy sampling and histopathological examination for correlated HPV pathologies and not HPV related. Counting of the total number of examinations where the presence of koilocytes or the presence of koilocytosis in the context of lesions affecting the oral mucosa are reported. 12 month period from October 2018 to October 2019.

4th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with an age division. Population studied and treated from October 2018 to October 2019.

5th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with a gender division. Population studied and treated from October 2018 to October 2019.

6th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with a division following a topographical criterion. Population studied and treated from October 2018 to October 2019.

7th Objective

Division by type of molecular diagnostic investigation method.

8th Goal

Division by type of serotypes identified.

RESULTS

1st Objective

In the course of 2018 and 2019, on the basis of the data we had previously collected, the recruitment of subjects with correlated non-HPV pathologies investigated through biopsy sampling from the afferent population and relating to the Oral Pathology and Medicine Clinic was carried out. and subsequent histo-pathological examination for the diagnosis of HPV pathologies related to the mucous membranes of the oral cavity (squamous papilloma, condyloma acuminata verruca vulgaris). The recruitment period lasted 12 months from October 2018 to October 2019.

In the corresponding period, 156 biopsies were performed (divided into incisional and excisional ones) with an average of three biopsies per week in patients visited for oral pathologies strictly correlated to the presence of HPV and for patients suffering from other pathologies.

From the analysis of the histo-pathological reports analyzed by the U.O. of Pathological Anatomy of the Polyclinic Hospital of Milan we had histopathological diagnostic findings and confirmations of pathologies directly related to HPV in 9 cases. The percentage stood at 5.76% out of a total of 156 biopsies diagnosing correlable HPV lesions. In particular, 9 cases of squamous papilloma were found.

Therefore:

• squamous papilloma number 9 5.76%

The values analysed.

Therefore:

• 156 biopsies

• 9 cases of HPV injury

• Percentage of HPV in biopsied lesions 5.76%

2nd Objective

As part of the cases of the Pathology and Oral Medicine Outpatient Clinic, the patients recruited were selected from subjects who had hyperkeratotic lesions of the oral cavity. These lesions were investigated through incisional or excisional biopsy sampling and subsequent histo-pathological examination in order to detect pathologies with the diagnosis of correlated non-HPV pathologies. At the level of the anatomical findings, the presence of histological signs of infection was evaluated (ie, as already described, the presence of koilocytes or the presence of koilocytosis). The patient recruitment period lasted 12 months, from October 2018 to October 2019.

In the corresponding period, 156 biopsies (incisional and excisional) were performed.

A total of 85 patients were recruited, whose biopsy samples were analyzed by the same U.O. of Pathological Anatomy of the Polyclinic Hospital of Milan and the presence of coilocytes or histo-morphological behaviours of koilocytosis were found at a histopathological level in 46 cases. The prevalence in these cases was around 29% with non-specific diagnoses of HPV lesions but of tissues with possible contamination (recent or past HPV) and with signs of contact with the virus.

Specifically:

• Leucoplakia number	1	0.6%
• Oral hyperkeratosis number	12	7.6%
• Oral lichen number	22	14.1%
• Proliferative villous leucoplakia number	2	1.2%
• Squamous cell carcinoma number	1	0.6%

Therefore:

• 156 biopsies

• 38 cases and with a percentage of 24.3% of non-specific diagnoses of HPV lesions but of tissues with koilocytosis.

3rd Objective

As part of the study of the data collected from the 156 biopsies performed, the data collected was counted and classified on the basis of the investigations carried out on biopsy sampling and histopathological examination for correlated and non-HPV correlated HPV pathologies where the presence of koilocytes is reported o the presence of koilocytosis in the context of lesions affecting the oral mucous membranes of patients recruited in the Oral Medicine and Pathology Outpatient Clinic, of the total number of subjects investigated through the Population studied and treated from October 2018 to October 2019.

In the corresponding period 156 biopsies (incisional and excisional) were performed

• we had 9 case reports with histopathological documented HPV related lesions. The percentage stood at 5.8%

• we had 38 case reports of non-HPV related pathologies but with the presence of koilocytes or histo-morphological behaviours of koilocytosis. The percentage was 24.3%.

• Total histopathological reports with signs of presence or contagion of HPV number 47 equal to 30.1%.

Data obtained testify to the widespread spread of the virus both in subjects presenting HPV-related tissue lesions but also in patients without apparent signs of HPV infection but who had contact with the virus in the presence of another type of pathological lesion.

This is an expression of the intrinsic characteristic of this virus of being also present in a purely asymptomatic form.

4th Objective

Of the 156 biopsies carried out, an attempt was made to analyse a correlation based on age. The literature has provided us with a grouping into specific age groups. The population was studied in the period between October 2018 and October 2019.

• Total of 156 biopsies

• 9 related HPV lesions. Percentage of 5.8%

• 38 case reports of related non-HPV diseases. Percentage of 23.4%.

• 47 reports of the presence or occurrence of HPV infection, equal to 30.1%.

Age groups considered and related to 9 related HPV lesions:

• age 0-6 0 0.0%

• age 7-12 0 0.0%

• age 13-20 0 0.0%

• age 21-40 1 11.1%

• age 41-60 1 11.1%

• age 61-70 3 33.3%

• age 71-80 3 33.3%

• age 81-99 1 11.1%

The results obtained show that the age group most affected is that of 60 and 80, age in which the presence of the virus manifested with the presence of related lesions, considering the latency period of the same of a few years. The injuries manifest themselves more in the later stages of life where human relationships have occurred previously.

Age groups considered and related to 38 reports of cases of non-HPV correlated diseases:

• age 0-6 0 0.0%

• age 7-12 0 0.0%

• age 13-20 0 0.0%

• age 21-40 0 0.0%

• age 41-69 23 7%

• age 61-70 12 31.6%

• age 71-80 16 42.1%

• age 81-99 1 2.6%

As evidence that the infections occur in the ages where there is greater exposure, ie from 40 to 80 years. The lack of lesions combined with asymptomatic pictures could be interpreted as inactive contagions in the silent phase of the virus within the cell that can be traced even after years through a histopathological examination for non-HPV correlated lesion.

5th Objective

This objective was aimed at reacting the data obtained with HPV correlated and non-HPV correlated pathologies with a division by sex. Population studied and treated from October 2018 to October 2019.

Breakdown by gender of 47 reports of presence or of HPV infection.

• Female sex 28 59.6%

• Male sex 19 40.4%

As evidence of a uniform spread of the virus and equally distributed in the two sexes. As reported in literature (Fig. 5-6)

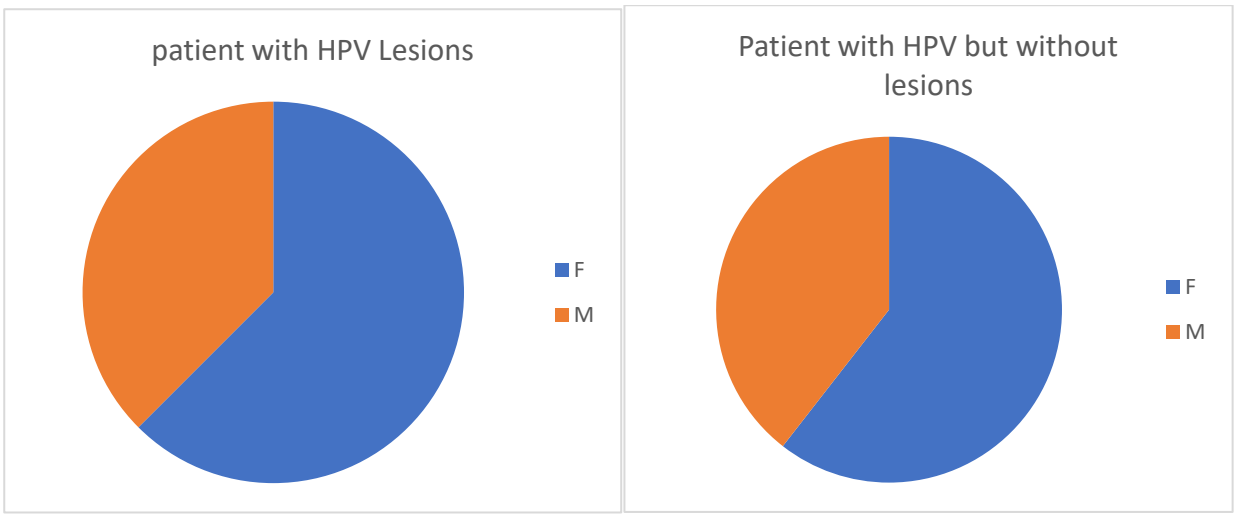

Fig. 5-6: distribution of HPV spread considering sex

6th Objective

Total number of subjects investigated for HPV-related and non-HPV-related diseases with a division following a topographical criterion Population studied and treated from October 2018 to October 2019.

9 HPV related injuries. Percentage of 5.7% of 156

• 38 case reports of related non-HPV diseases. Percentage of 24.3%. of 156

Topographical criterion which includes:

• anterior regions of the mouth,

including the lips and anterior vestibules

• middle regions of the mouth

at the end of the hard palate and at the anterior region of the lingual V

• posterior regions of the mouth

including oro-pharynx.

Topographic criterion applied to 9 related HPV lesions (fig.7)

• anterior regions of the mouth 5 56%

• middle regions of the mouth 2 22%

• posterior regions of the mouth 2 22%

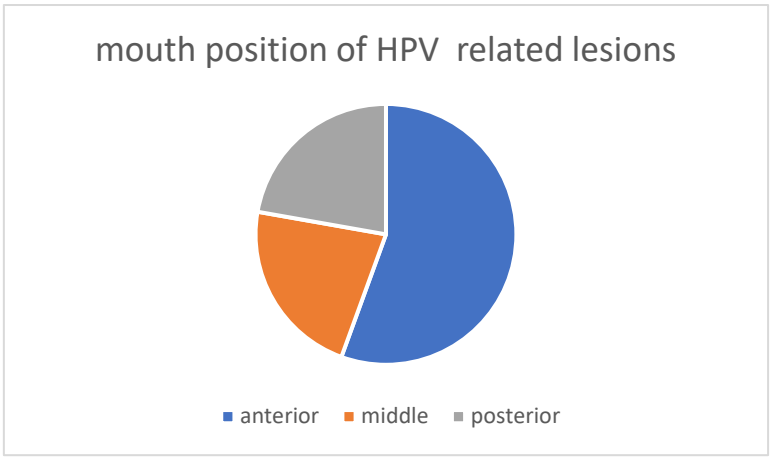

Fig.7: HPV related lesion oral position

Topographic criterion applied to 38 unrelated HPV lesions (fig.8)

• anterior regions of the mouth 13 34%

• middle regions of the mouth 22 58%

• posterior regions of the mouth 3 8%

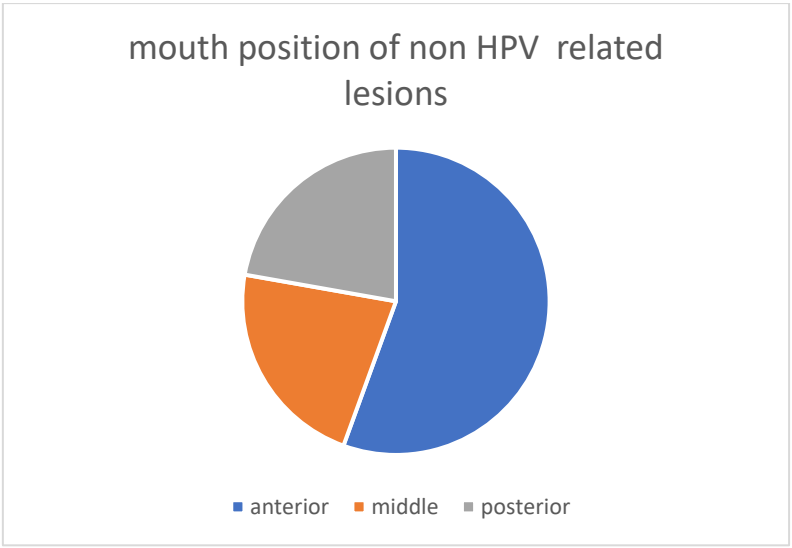

Fig.8: HPV not related lesion oral position

Results testify to the spread of viruses not particularly related to AC. oral and not often linked to diffusion for sexual practices.

The presence of the virus, in the posterior regions of the mouth, on the other hand, testifies to the presence of strains that do not manifest themselves with clinically objectionable pathologies but

traceable even after years of permanence and more expression of sexual practices. In fact, the tonsillar epithelium represents an ideal growth medium for the nesting of the virus that can remain silent for years and initiate the cellular transformation typical of its presence, which are koilocytes.

7th Objective

All our biopsies were investigated using this molecular diagnostic method by the U.O. of Virology and Serodiagnosis of the Cà Granda IRCCS Foundation Hospital of Milan.

A viral genome sequence is first amplified by PCR. The amplicon obtained is denatured and detected by hybridization with specific probes immobilized on strips, filters or micro-plates and colorimetric or chemiluminescence reaction. In vitro PCR reconstructs a specific step of cell duplication: the reconstitution (synthesis) of a "complete" (double-stranded) DNA segment starting from a single-stranded strand. The missing strand is reconstructed starting from a series of nucleotides, the elementary "building blocks" that make up the nucleic acids, which are arranged in the correct sequence, complementary to that of the affected DNA.

This process is carried out in nature by enzymes called DNA-polymerases, which are able to progressively synthesize a new DNA strand under the following conditions:

• the nucleotides to be polymerized must be available in the form of deoxy-ribonucleosides-triphosphates (dNTP);

• the DNA must be denatured, ie the two helices that make up the strands must already be separated;

• the segment to be reconstructed can only be prolonged, ie it is not possible to synthesize a new strand starting from zero;

• appropriate conditions of temperature, pH, etc. must also be observed.

It is therefore possible to reconstruct the conditions that lead to the formation of new DNA segments, by putting in solution:

• even a small amount of the segment of DNA to be reproduced;

• an appropriate quantity of free nucleotides to make up the new filaments;

• suitable "primers", called primers, consisting of short DNA sequences (oligonucleotides) complementary at the 5 'and 3' ends of the two filaments of the segment to be reproduced;

• a thermo-resistant DNA polymerase (it does not need to come from the same organism whose DNA is to be replicated);

• a buffer that serves to keep the pH suitable for the reaction stable;

• other supporting elements, such as magnesium ions, essential for the correct functioning of DNA polymerase;

To start the polymerase reaction (phase of extension of the filament starting from the primer 5 ') it is first necessary to provide for the separation of the DNA strands (denaturation phase), then to create the bond between the primers and their complementary regions of the filaments of denatured DNA (annealing phase). However, this process is incompatible with human DNA polymerase, which is destroyed at the temperatures necessary for denaturation (96-99 C).

To overcome this drawback, use is made of polymerases belonging to thermophilic organisms that are not inactivated by high temperatures, for example the Taq polymerase coming from the thermophilic bacterium Thermus aquaticus. This allows to carry out several PCR cycles in sequence, in each of which the DNA synthesized in the previous phases is also duplicated, obtaining a chain reaction that allows an extremely rapid multiplication of the genetic material of interest.

Subsequently, the method called LIPA (linear probe access) uses probes linked to a nitrocellulose membrane that "capture" any specific DNA sequences.

This method is based on the link between biotin and streptavidin and consists of three steps:

1) The biotin-labeled amplified DNA is hybridized with the probe

2) the biotinylated hybrid is incubated with streptavidin linked to the enzyme

3) the substrate is put in contact with the DNA-biotin-streptavidin-enzyme complex and develops a colorimetric reaction.

As part of our research, over the 12-year period, the data refer to laboratory investigations of the U: o: Pathological Anatomy alone, where methods such as:

• Broad spectrum PCR

• type-specific PCR

The use of broad-spectrum primers appears to be the most used approach. In this case the primers target a highly conserved region of the viral genome in the different HPV types, in order to recognize a broad spectrum of genotypes. The most suitable genomic region in this sense is the L1 region, although primers that map to the E1 gene have been described in the literature.

Type-specific primers allow detection of the presence of a single viral type per reaction. This approach is therefore useful if a specific viral type is investigated or suspected eg. HPV-16 in oral cancer.

At our pathological anatomy, broad-spectrum investigations and specific investigations for HPV 16 are carried out at our routine request.

• In the context of the research of this last period, the diagnostic collaboration with Pathological Anatomy has expanded with the U.O. of Virologists Virology and serodiagnosis of the Polyclinic Hospital of Milan Fondazione Cà Granda IRCCS;

• in Virology and serodiagnosis of the Cà Granda IRCCS Foundation Hospital of Milan;

Specific tests have been applied at the Virology facility that allow genotyping of all types of HPV. Most of the tests included in this category use the reverse hybridization method of a broad spectrum of HPV types. A viral genome sequence is first amplified by PCR, the amplicon obtained is denatured and detected by hybridization with specific probes immobilized on strips, filters or micro-plates and colorimetric or chemiluminescence reaction. In this case the LIPA test was used.

8th objective

Analyzing the results of studies conducted in various parts of the world, the IARC (International Agency for Research on Cancer) has more recently officially identified types 16, 18, 31, 33, 35, 39,

45, 51, 52, 56, 58, 59 and 66 as carcinogens, while HPV types 6 and HPV 11 are considered possibly carcinogenic to humans while HPV types 6 and HPV 11 are considered possibly carcinogenic to humans.

Classification based on the Tropism of the virus: they are divided into two broad categories, cutaneous HPV and mucosal HPV. Cutaneous HPVs cause skin lesions such as common or vulgar warts (they are the most common forms), they are frequently localized on the hands and feet; another part of cutaneous HPV types are associated with verruciform epidermodysplasia.

Mucosal HPVs cause different types of lesions affecting the genital tract in both women and men; HPV 6 and HPV 11, belonging to the low-risk types, lead to the formation of acuminate warts (benign warts), while the high-risk types are found in squamous intraepithelial lesions that can progress to invasive squamous carcinoma.

Of the 47 samples in which histo-morphological characteristics of koilocytosis or koilocytes were identified, 28 were found to be typable by the LIPA method and in 19 cases the genotype 16 was detected, which is considered to be at high risk. (Fig.9)

Genotype 16 13 28.26%

Genotype 16 + 33 3 6.52%

Genotype 16 + 45 3 6.52%

Genotype 6 6 13.04%

Genotype 6 + 44 3 6.52%

Not Typeable 19 39.13%

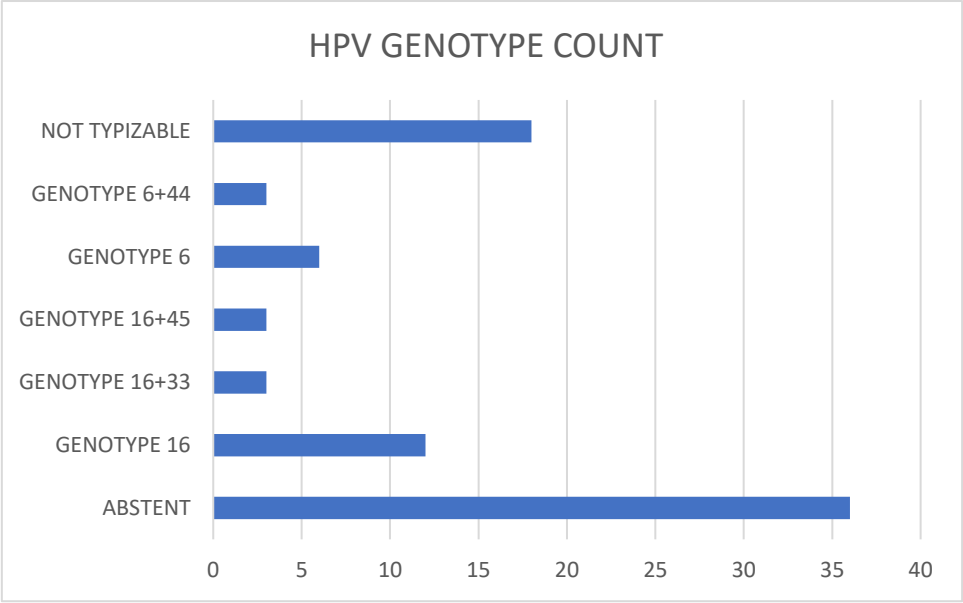

Fig.9 HPV genotype count

The distribution of genotypes in relation to gender indicates a lower spread of genotype 16 in male patients (30%) than in females (48.1%). The frequency of genotype 6 is higher in males (25%) than in females (14.8%). (tab 2) (fig.10)

	MALE	MALE %	FEMALE	FEMALE %
Genotype 16	2	10,0	11	40,7
Genotype16+33	1	5,0	0	0,0
Genotype16+45	3	15,0	2	7,4
Genotype 6	4	20,0	2	7,4
Genotype 6+44	1	5,0	2	7,4
Not Tipizable	9	45,0	10	37,0

Tab.2: distribution of genotypes in relation to gender

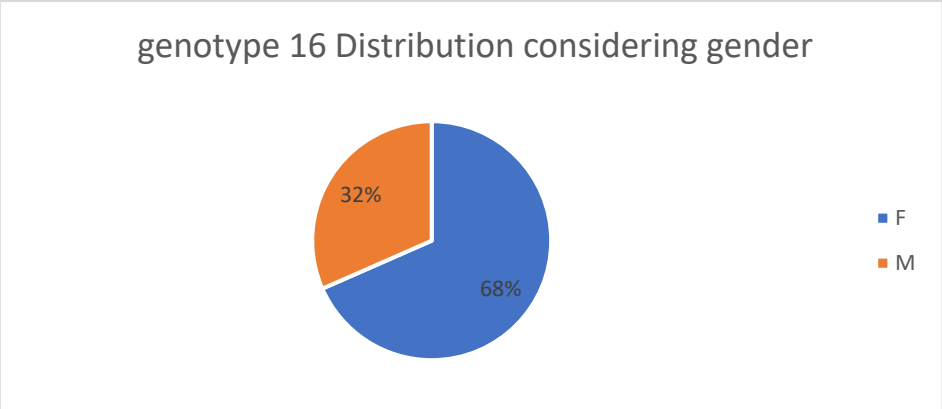

Fig. 10: Distribution of HPV in gender

Furthermore, the distribution of genotypes in relation to depth in the oral cavity was verified (fig.11)

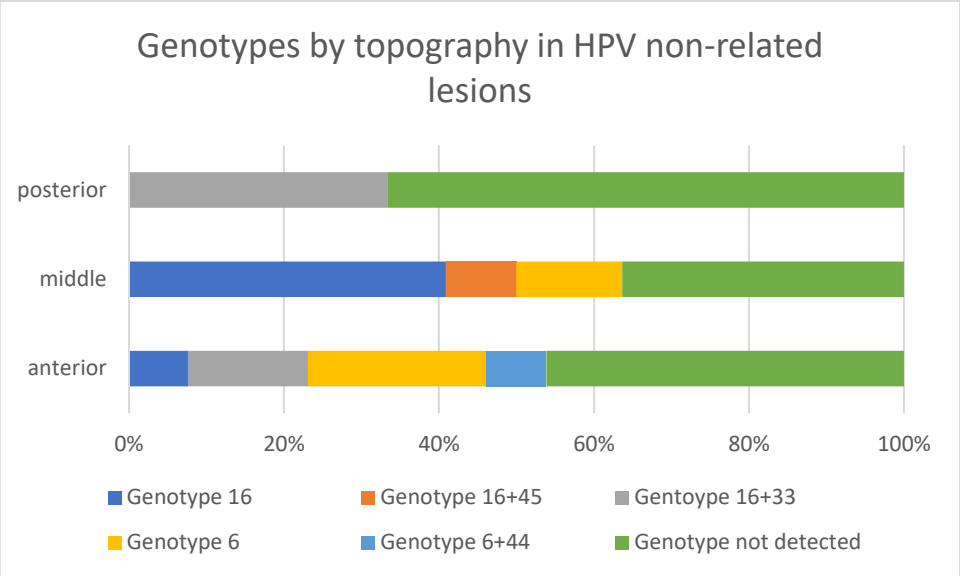

Fig.11:genotypes by topography in HPV non related lesions

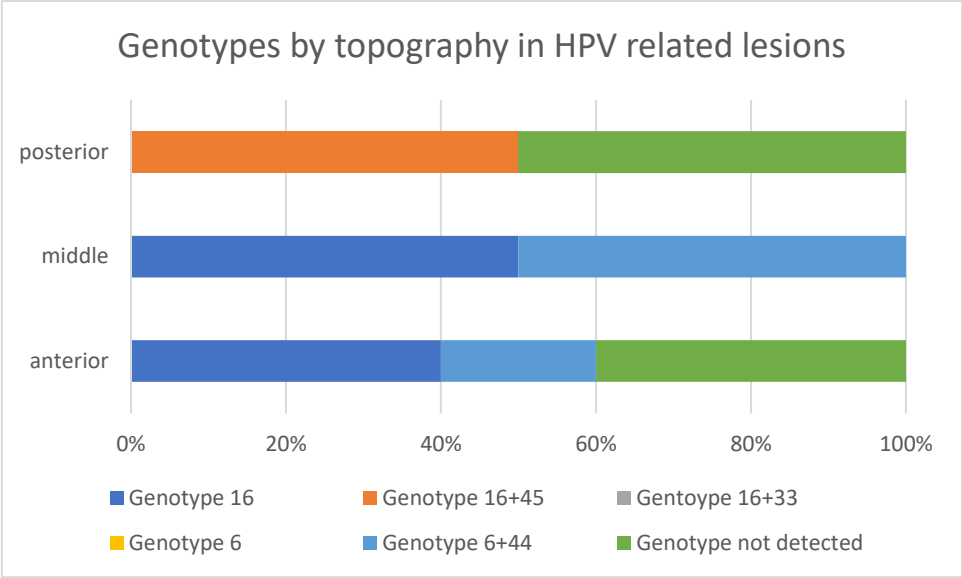

Fig.12:Genotypes by topography in HPV related lesions

Genotype for depth in the oral cavity

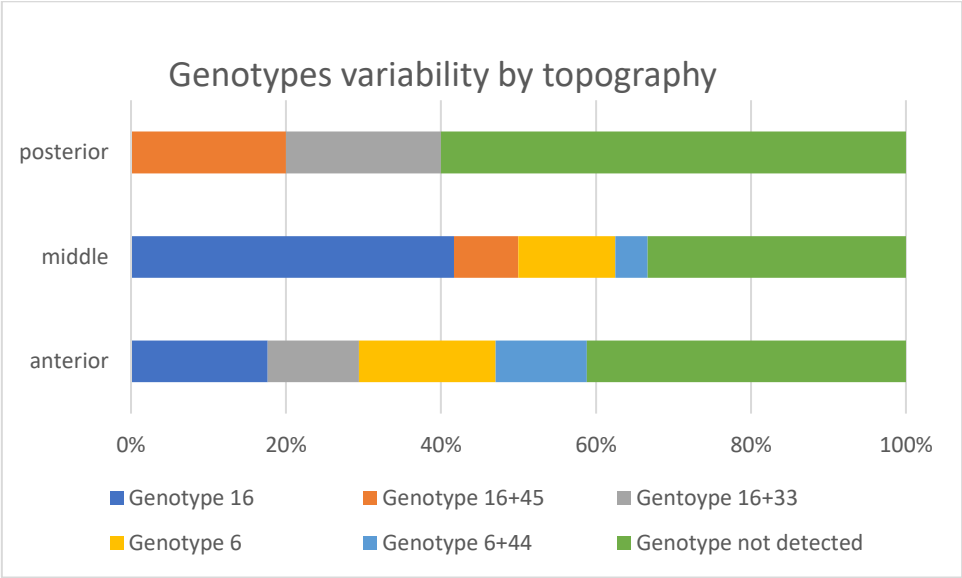

Fig.13:Genotype variability by topography

In overall terms, the distribution of viral genotypes as a function of the topography of HPV + lesions have the following differences:

• The share of viral DNA that cannot be typed with the LIPA technique is one third for lesions located in the anterior and middle portion of the mouth. This share increases in the posterior regions, where a percentage of untypable genotypes equal to 60% is found

• Genotype 6 is mostly represented in anterior (30%) and medium (17%) lesions

• Genotype 16 is represented in 50% of lesions with medium localization, in 40% of posterior lesions and in 30% of anterior lesions

Therefore, there is a greater variability in the genotypes typed by LIPA in the anterior and middle lesions, which in a third and in a quarter of cases respectively host genotype 6. The range of typable genotypes decreases proceeding in the posterior portions of the mouth and increases in percentage terms the frequency of genotype 16.

Chapter 5

Discussion and conclusions

The study of the papillomavirus genome is fundamental for the classification of the virus and for the analysis of phylogenetic correlations between the different viral types. The phylogenetic evolution of the virus certainly has repercussions on the evolution of the biological capabilities of the different viral types. They seem to have evolved to occupy different biological niches, becoming specific species, acquiring the ability to infect different anatomical districts, while all being epitheliotropes. To better understand how this is possible, it is essential to study the molecular mechanisms and how the different viral types interact with the cell. In this way it is possible to understand how the differences in the genome are expressed on the viral mechanisms and how some phylogenetically distant viruses can give similar lesions and neighboring types different lesions. However, genotyping also has enormous clinical and epidemiological importance.

Persistent high-risk HPV infection is recognized as a necessary cause of cervical or oral-pharyngeal cancer. With the implementation of a prophylactic HPV vaccination program, an accurate typing method was required to evaluate vaccine efficacy and to monitor the distribution of HPV types in the general population and vaccinated cohorts (Ferguson et al. , 2009). In addition, HPV typing has been introduced into the cervical cancer screening program as a second line test for the management of high-risk HPV-infected women (Barzon et al., 2008; Saslow et al., 2012).

Worldwide, the prevalence of HPV infection in women ranges from 2% to 44%. This variability is attributable to differences in the age of the population studied, geographical area and sensitivity of the test used to search for HPV infection. However, it is estimated that more than 50% of sexually active women have come into contact with one or more viral types, while little is known about the infection in men (Baseman et al., 2005). Since oncogenic potential varies between different high-risk HPV types, HPV genotyping is to be considered for the management of women with high-risk HPV infections.

The definition of the oncogenic potential of a single viral type is mainly based on epidemiological evidence of association between the identification of HPV DNA and invasive carcinoma (58-69-15) and depends on the accuracy of the methods HPV identification and genotyping. In this regard, the

WHO Global Proficiency Studies demonstrated the need to improve methods for reliable HPV genotyping, given that many false positive and false-negative results were reported by participating laboratories (71).

The biomolecular method that involves the use of PCR in the diagnosis of HPV infections, it is extremely useful because this technique is rapid and of easy to perform and, above all, because it is extremely sensitive. Furthermore, the HPV genotyping that is carried out in case of positivity for the genome HPV is an excellent means of prevention / control for cervical cancers uterine, as genotypes associated with pathologies can be highlighted neoplastic. The application of this method, in our study, has allowed us to achieve some interesting results. We were able to demonstrate how the infection is extremely widespread in our series, the use of PCR probably played a role key. This method is turned out to be fundamental thanks to its accentuated sensitivity that allows amplify viruses present in the sample in low quantities and detect a large number of different HPV genotypes. From this it follows as the identification of the HPV infecting genotype is an essential diagnostic necessity and an important tool for the detection of cervical cancer. Research performed shows that HPV sequences of high types risk, are almost always present in cases of ASCUS associated with CIN2, while negative cases are rarely associated with injury. The use of the Molecular typing serves to assess the presence of infection in a range of people undergoing screening as a marker of risk of progression (especially for genotypes 16 and 18). This with regard to studies epidemiological to establish the prevalence of the different types. On the basis of these premises, the application of the PCR method we put in place point for the detection of infection and subsequent genotyping using inno-LiPa method, should provide relevant statistical data to be included in broader research fields. The same are aimed at the production of data conclusive that confirm the applicability of the method in programs mass screening, with an attractive cost-benefit ratio, but not only: molecular biology allows us to study the most relevant genotypes since clinical point of view starting from a single amplification product, with the security given by a real gene sequence.

Since the causal link between the infection with HR-HPV and cancer development has been well established, there is a general consensus that HR-HPV DNA testing could increase the efficacy of the present cytologic screening programs (18) and recently established guidelines recommend HR-HPV DNA testing in order to improve the efficacy of primary cytologic screening programs or triage tests (16-44-70).

In the course of this research we wanted to look for a method that would allow us to detect the presence of HPV that can be correlated or not with lesions within the oral cavity. This presence was supposed to be silent, ie present but without clinical manifestations, or present with typical lesions. The first phase of the study required a detailed analysis of the medical records and the archive of the Oral Pathology Outpatient Clinic of the IRCSS Ca 'Granda Hospital in order to detect data such as oral cavity lesions, age of patients, sex, topography of lesions and virus genotyping in lesions.

The population investigated was that of patients in the clinic with the presence of related and non-related HPV lesions. Therefore, this study could not analyze healthy patients, but its aim was to establish the potential of a study applicable on a larger scale and therefore extendable as a screening method to the entire population.

After a first review of the case series from 2005 to 2016, the active phase of the study proceeded, i.e. the recruitment of patients and the study of the same thanks to biopsy analysis first and molecularly thereafter.

We wanted to search for the same data that the previous exploratory phase had provided us in order to be able to perform a primary comparative examination. In particular, the type of lesion, the presence or absence of HPV, the age of the patient, the sex, the lesion topography and the HPV genotype detected when possible were studied.

After the collection of these data, a comparison was made between these and those obtained from the first phase. It was shown that in both researches the age of detection of the presence of the virus was between 20 and 80 years, which represents the age group where inter-human relationships are most represented. In the samples of the second phase it was also found that the development of the related

HPV lesions took place between 60 and 80 years, making us suppose that the period was related to the fact that the virus can remain latent for years before developing the full-blown disease.

There was no difference between the two research phases as regards the distribution on the basis of sex. Men and women were equally affected, there was only a variation of the HPV genotype detected: The distribution of genotypes in relation to sex testifies to a lower spread of genotype 16 in male patients (30%) than in females (48.1%). The frequency of genotype 6 is higher in males (25%) than in females (14.8%).

The topographic survey of the genotype revealed a greater presence of the virus at the level of the anterior part of the mouth considering both the related and unrelated HPV lesions while the posterior and middle parts reach different percentages, particularly in the first phase of the study it was seen that the virus was more represented in the medial part for related HPV lesions while in the posterior part the percentage was higher than in the other areas for correlable non-HPV lesions in our active research it was seen that In overall terms, the distribution of viral genotypes as a function of topography of HPV + lesions has the following differences: The share of viral DNA that cannot be typed with the LIPA technique is one third for lesions located in the anterior and middle portion of the mouth. This share increases in the posterior regions, where there is a percentage of non-typable genotypes equal to 60%, Genotype 6 is more represented in lesions with anterior (30%) and medium (17%) localization Genotype 16 is represented in 50% of lesions with medium localization, in 40% of posterior lesions and in 30% of anterior lesions

Therefore, there is a greater variability in the genotypes typed by LIPA in the anterior and middle lesions, which in a third and in a quarter of cases respectively host genotype 6. The range of typable genotypes decreases proceeding in the posterior portions of the mouth and increases in percentage terms the frequency of genotype 16.

In conclusion, genotyping in the study of cancer is useful to provide further data on the causal relationship between cancer and viral infection, on oncogenic HPVs and on the cellular modifications they induce. On the vaccinated population, it allows to monitor the duration and effectiveness of

protection and is useful in stratifying HPV positive women based on the risk of developing precancerous or cancerous lesions and thus to guide clinical decisions (46).

As highlighted in section 3 the genotyping tests in use today differ in their analytical capabilities for sensitivity and type specific specificity and many studies have compared different HPV typing methods using different clinical samples. In the vaccination era, a high analytical sensitivity is required, as a failure to identify the prevalent infections upon entry into a trial can lead to a false result of vaccine failure in vaccination protocols.

The will of this initial study, which will progress beyond the conclusion of this path, is to evaluate a rapid, effective and feasible method on a large scale to extend the research of HPV even in patients not suffering from overt correctable HPV pathologies in order to plan a screening. for the follow-up of the development of related HPV oral cancer.

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