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ROLE OF EXOSOMES IN THE TRANSFER OF VIRAL NUCLEIC ACIDS TO RECIPIENTS CELLS: IN VITRO STUDIES WITH CELL LINE SUPERNATANT AND PATIENT-DERIVED EXOSOMES

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1. INTRODUCTION

1.1 BREAST CANCER

1.1.1 MAMMARY GLAND

The mammary gland begins to form in the sixth week of fetal development, becoming a complex structure in women, relative to men. Its development in women is not complete but it continues to change during adolescence and pregnancy.

The mammary gland is made up by a differentiated parenchyma supported by fibrous scaffold, the stroma, surrounded by fatty tissue. The breast glandular tissue is made up of a complex network that looks like resembles a bunch of grapes. In adults each breast is composed of about 15 to 20 lobes. Each lobe consists of several saccular structures called lobules, which proceed towards the nipple through channels called milk ducts, which in turn dilate forming the lactiferous sinuses and branching further distally form the terminal ducts. Before puberty this ductal branches system ending dead-end, but at the time of menarche is developed resulting in 30 epithelial units, bile or berries. Bile-duct with its terminal constitutes the ductal-terminal lobular units. In the female puberty begins a branching process stimulated by GH (growth hormone), pituitary, estrogen, progesterone, synergistically with IGF-1 (insulin-like growth factor-1) and with EGF (epidermal growth factor). Breast reaches its full morphological maturity and maximum functional activity only with the pregnancy. Gland is almost exclusively made up of glandular structures separated from each other by thin stromal bands at the end of pregnancy. Immediately after giving birth begins the secretion of milk and after feeding, the glands undergo atrophy, the ducts are reduced and the volume of the breast regresses significantly (Stingl J et al., 2007) (Figure 1).

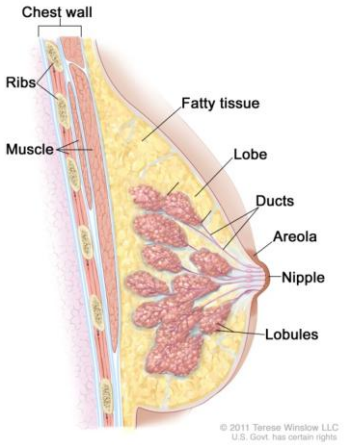


Figure 1: Breast structure image (National Cancer Institute)

1.1.2 EPIDEMIOLOGY

Breast cancer is the most common cancer in women and it is the leading cause of death worldwide. It is particularly present in most developed countries, especially America, Australia, Europe, New Zealand. In 2008 it was estimated that breast cancer accounts for 23% of all diagnosed cancers and 14% of all cases of death from cancer (Jemal A et al., 2011; Italian Cancer Registry). Recently, there was a new trend concerning the increased incidence of breast cancer even in less developed countries. It is estimated that 45% of the 1.35 million new cases diagnosed each year, and more than 55% of breast cancer related deaths, occur in low and middle income countries (Curado MP et al., 2007; Porter P, 2008; Tfayli A et al., 2010).

1.1.3 RISK FACTORS

The development of breast cancer has been associated to various risk factors. As previously said, the geographic location is an important component for the risk of developing cancer. In industrialized countries, the rates of developing cancer are higher than in underdeveloped countries, although recently, it seems there is also a rise in those populations (Porter P, 2008).

The family and genetic component, play an important role in breast cancer development. One or more cases of breast cancer in family increase the risk of tumor development, whereas mutations in BRCA1 (17q) and BRCA2 (12q) genes correlate with a greater chance of developing cancer within 70 years.

Hormonals factors are very important. Several studies have shown that many hormones and growth factors are associated with the risk of developing breast cancer. (Key TJ et al., 2003; Kaaks R et al., 2014).

Behavioral component is not to be underestimated: alcohol and smoking increase the risk of onset of breast cancer (Xue et al., 2011; Key J et al., 2006). Furthermore, it has been shown that a low-fat diet reduced 8% the risk of breast cancer (Prentice RL et al., 2006).

Obesity is to be kept under control especially in the period before and after menopause, probably because in the overweight women occur a steroid hormones increased that could lead to a major risk of developing cancer (Youlden DR et al., 2012; Colditz GA, et al., 2014; Renehan AG et al., 2008).

Finally, women who exercise basic sport activity, are less subject to develop breast cancer (Youlden DR et al., 2012).

1.2 CLASSIFICATION OF BREAST CANCER

1.2.1 BENIGN DESEASE

Breast cancer consists of an uncontrolled proliferation of epithelial cells originating from the breast ducts or lobules. The tumors are divided into benign and malignant forms, with a greater frequency of benign forms.

Some of benign forms may resemble clinically to malignant forms, while others may be a possible risk factors for cancer development.

The most frequent benign form is fibroadenoma, a fibrous and glandular tissue proliferation, which occurs most often in young women (15-30 years).

The fibroadenoma is characterized by a nodule with a smooth surface and a variable consistency: in some cases, can be rubbery; in others, it can be hard; other times, it is described as a highly mobile ball. The fibroadenoma can be simple or complex. In the first case, the size and shape remain unchanged and over time can reduce its volume. For this reason it doesn't present a particular problem. The complex fibroadenoma, may contain abnormal dense cysts, or calcifications, and should therefore be kept under periodical observation. The appearance of fibroadenoma can be single, but in some cases may be multiple or bilateral. The causes of fibroadenoma formation, have not yet been clarified completely. The origin seems linked to the excessively high levels of estrogen. This association explains high incidence of fibroadenoma formation among pregnant and young women, where there is a significant amount of circulating estrogen to the body, and the low incidence among menopausal and post-menopausal women, where the number of estrogen is significantly reduced.

Intraductal papilloma is another breast benign lesion. It has been found in young and middle-age women (35-50 years). It's a very small size mass, formed by fibrous and glandular tissues and blood vessels. It is generally formed in the retro-areolar milk ducts that are responsible for secreting milk. The most common types are: solitary intraductal papilloma occurs in a mammary duct, usually near the nipple; multiple papillomas, appears in the cluster form often in the most peripheral ducts and in some cases may evolve into a breast cancer. The intraductal papilloma is one of the most common causes of nipple discharge, which overall is found in about 5% of women with breast pathologies.

Fibrocystic disease is the most common benign condition. It has been found in 60-90% of women, most frequently between 20-40 years. It consists in alterations of morphological mammary tissue, such as fibrosis, chronic inflammation, epithelial hyperplasia and the presence of cysts containing clear fluid or blood. Normally it is bilateral and involves the breasts widely; but it can also be unilateral preferring superior-external area. Its origin is linked to the production of different hormones in the various phases of the cycle. It is assumed that there is also a certain glandular congenital predisposition, destined to manifest itself in the fertile period, from puberty to menopause.

1.2.2 MALIGNANT DESEASE

Breast cancer is divided in histological and molecular classification. The histological classification, described morphology (lobules or milk ducts cells alterations), invasiveness (invasive or non-invasive) and grades or stages of tumor (0 to 5). In the molecular classification, tumor can be characterized by hormonal receptors presence. Therefore, we distinguish four different molecular subtypes: Luminal A, Luminal B, Triple Negative / Basal-like subtype Her2.

1.2.2.1 HISTOLOGICAL CLASSIFICATION

NON-INVASIVE FORMS

Ductal carcinoma in situ

Ductal carcinoma in situ (DCIS) is a less aggressive non-invasive form of breast cancer. The tumor is confined into the duct without invading the surrounding tissues. It can be divided into low (Grade 1), intermediate (Grade 2) and high (Grade 3) grade. DCIS is categorized according to the growth of ductal cells mode: “comedonico”, characterized by irregulars and large cells, with high proliferation and necrosis of the duct central part; and "non-comedonico", said also cribriform / micropapillary, composed by small and regular cells, free of necrosis.
If DCIS is not taken care, the confined cells in the duct, can invade surrounding tissues, becoming an invasive ductal carcinoma.

Lobular carcinoma in situ

The lobular carcinoma in situ (LCIS) is a precancerous: cancer cells grow inside the lobules and remain "in situ" and do not extend outside of them. This feature makes LCIS prognosis very good. LCIS is more frequent among 40-50 years: in 40-85% of cases is multicentric and in 30% of cases is bilateral. It is associated with an invasive form in 5% of patients

INVASIVE FORMS

Invasive ductal carcinoma

Invasive ductal carcinoma is representing 70-80% of all forms of breast cancer. It originates from the milk ducts, then exceeds the duct walls, penetrates in breast adipose tissue and may invade surrounding tissues or spread to other parts of the body.

Invasive lobular carcinoma

Invasive lobular carcinoma is representing 10-15% of all forms of breast cancer. It originates from the lobules and overcoming the basal membrane, can spread and invade other body areas. It can appear simultaneously in several points, either in the same gland, that in both glands.

OTHER FORMS

Medullary carcinoma

Medullary carcinoma is representing for 5% of all breast cancers. It is characterized by a massive tumor, with well-defined contours. In most cases there is an abundant lymphocytic infiltration. The infiltration intensity has favorable prognostic significance, because it represents an expression of tumor host response.

Inflammatory carcinoma

The inflammatory carcinoma is a breast cancer rare form (1-4% of all cancers) and is a fast-growing tumor. It may resemble some forms of breast infections (mastitis). Mammary tissue appears red and

inflamed, due to the block of breast and skin lymph vessels by cancer cells. Breast changes can happen very quickly.

Paget's desease

Paget's disease is a rare form of breast cancer (<5%) that develops in the ducts cells and spreads to the nipple and areola. It has a nipple reddened flaking and may spread to areola, causing itch, sting and discharge. It is associated with invasive ductal cancer when there is a lump. This condition occurs in approximately half of cases.

Mucinous carcinoma

The mucinous carcinoma, also said colloid, represents about 2% of all breast tumors. The name is due to the mucus surrounding the tumor cells. It has a better prognosis and a lower risk of metastases.

Tubular carcinoma

Tubular carcinoma is very rare tumor. It represents 2% of all breast cancers and is more common in women over 50 years old. It is characterized by the tubular tumor cells presence. It can be associated with infiltrating ductal carcinoma or remain pure with a better prognosis.

Cribiform carcinoma

About 4% of breast tumors contain a part cribriforme. It is characterized by gaps between the cells that make it look like a sieve. Often the cribriform carcinoma is associated with tubular carcinoma.

Malignant phyllodes tumor

Malignant phyllodes tumor is a rare kind of tumor (<1% of all cancers) that affects women with 40-50 years not yet in menopause. It appears as a smooth, hard lump that can grow rapidly and also it become large. It can present three forms: benign, borderline and malignant.

Papillary carcinoma

Papillary carcinoma is a rare kind of tumor, accounting for 1% of all cancers. The cells take on a particular fern shape, from which it derives the name.

1.2.2.2 MOLECULAR CLASSIFICATION

Breast cancer is characterized by its considerable molecular heterogeneity. The tumor presents different mutated genes, whose only some of which are protagonists of the tumor process (Lopez et al., 2009). For this reason, patients with identical histologically breast cancer, may have a different disease course and a different response to the therapies used.

Thanks to the use of microarrays (Sorlie T et al., 2001; Sorlie T, 2004), it was possible to classify breast cancer at the molecular level. The molecular classification is divided into:

Luminal A: malignancies with hormone receptors expression, HER2 negative and low proliferative activity;

Luminal B: tumors with hormone receptors expression. It is associated with a high risk of recurrence, due to the high proliferative index correlated to high expression of proliferation genes;

Basal like/ Triple negative: malignancies characterized by the absence of hormone receptors and HER2 expressions. It is characterized by a cytokeratin (CK5 / 6 and CK 17) increased expression.

HER2 subtype: tumor characterized by the presence of HER2 and by increased of genes involved in the HER2 expression.

Thanks to the new pathological and molecular knowledge there is a definition of additional breast cancer subtypes. Recently it has been identified, another subgroup of tumors with no expression of hormone receptors and HER2, but with markers of stem cells, low expression of claudine (cell-cell junction proteins) and lymphocytic infiltrate, called "claudin low" and characterized by poor prognosis (Carey LA, 2010; Prat A et al., 2010; Malhotra GK et al., 2010). In addition, gene expression analysis of 587 triple negative breast carcinomas has identified six different subtypes distinguished by a different molecular biology and a different clinical behavior: basal like 1 e 2 (BL1 e BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL) and luminal androgen receptor (LAR) (Lehmann BD et al., 2011; Ahn SG et al., 2016). The creation of cell lines derived from each subtype has also allowed to show a different sensitivity to chemotherapeutic agents and targeted therapies (Carey LA, 2010; Lehmann BD et al., 2011; LM_AIOM_MAMMELLA, 2015).

2. PAPILLOMAVIRUS

2.1 CLASSIFICATION

Papillomavirus (HPV) is a virus of Papovaviridae family. HPV has been discovered both in human and animals. More than 300 HPV have been identified and completeley sequenced, including over 200 human HPV (Egawa N et., 2015; Van Doorslaer K et al., 2017). HPV is a species-specific virus with a high tissue tropism: It has the ability to infect the epidermis and mucosa epithelial tissues, generating specific lesions. In this regard, the HPV genotypes were divided into two distinct groups: cutaneous and mucosal. The cutaneous HPV are associated with lesions (warts) on the hands and feet (1,2 and 4 genotypes), while other HPV genotypes are associated with epidermodysplasia verruciforms (5,8,9,12,14,15,17,19-25,36,46 and 47 genotypes). 5 and 8 HPV genotypes are associated also with a skin cancer (Cardoso JC et al., 2011; Orth G, 2008; Lazarczyk M et al., 2009).

The mucosal HPV, give lesions of the oral, respiratory and anogenital tract. They are divided into low and high risk, according to their oncogenic capacity. Among low-risk genotypes, 6,11,40,42,43,44,54,61,70 and 72 genotypes are classified as a low-risk genotypes, that can lead to genital mucosal lesions benign. 16,18, 31, 33, 35, 52, 58, 67 genotypes are considered high risk; HPV16 genotype is most frequently associated with cervical cancer (Stoler, 2000).

The use of phylogenetic algorithms, able to compare multiple genomic sequences, allowed to divide HPV genotypes in 37 genera. It was obtained analyzing the sequence homology existing between the nucleotide sequences of the L1 ORF region of the genome (Van Doorslaer K, 2013; Bernard HU et al., 2010; de Villiers EM et al., 2004).

Human HPV belong to 5 genera: Alpha, Beta, Gamma, Mu and Nu, which correspond to different types having life cycle and disease associations different (Bernard et al.,2010; De Villiers EM et al., 2004, Van Doorslaer K et al., 2013; Doorbar J et al., 2012) (Figure 2).

HPV is considered a new genotype when the homology existing between its DNA sequences and those of the already classified genotypes not exceed 90%. Viruses with a homology greater than 90% but less than 98% are defined subtypes and those with a homology between 98% and 100% are defined variants (De Villiers EM et al., 2004).

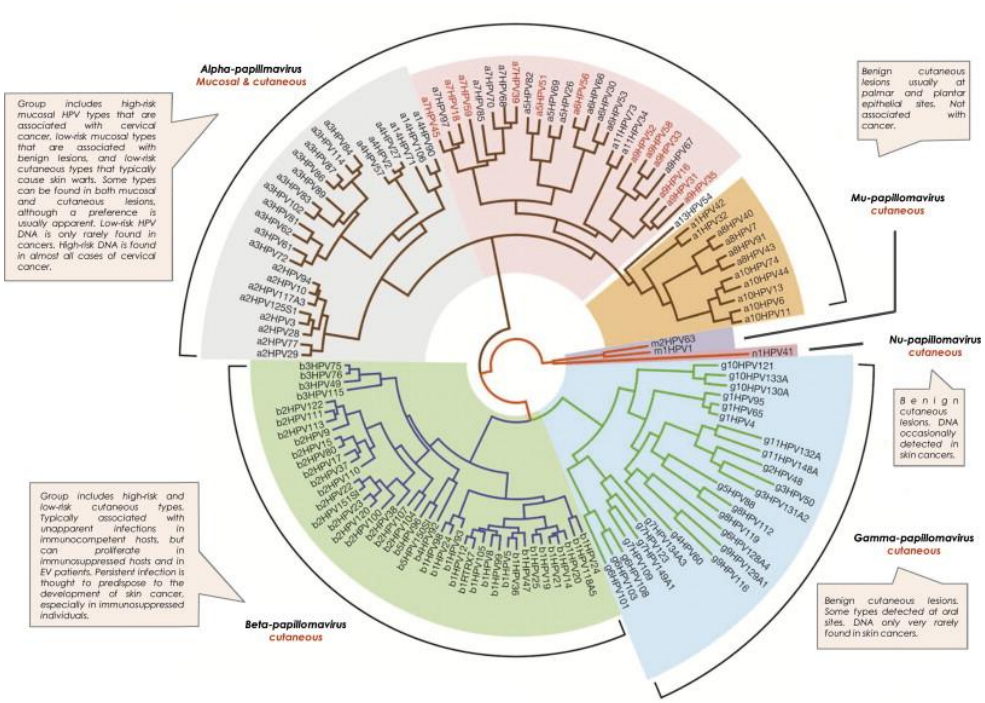


Figure 2: HPV genotypes genera (Egawa N et al., 2015)

2.2 GENOME ORGANIZATION

HPV is a circular double-strand DNA virus of about 8000 base pairs with a non-enveloped icosahedral capsid. It contains three distinct regions: late (L), early (E) and upstream regulatory region (URR) or LCR (long control region). L region includes two genes: L1 (major viral capside) and L2 (minor viral capside), which encode structural proteins that are necessary for viral capside formation in the final stage of replication. E region contains seven genes expressed in the early phase of the replicative cycle, which encode for non-structural proteins, with various regulatory functions called E1 to E7. L and E regions are separated by URR, a region of about 1000 base pairs that contains a number of cis elements necessary for the regulation of gene expression, DNA replication and genome viral encapsidation (Figure 3).

URR is a non-coding region, as opposed to E and L regions that present open reading frame (ORFs).

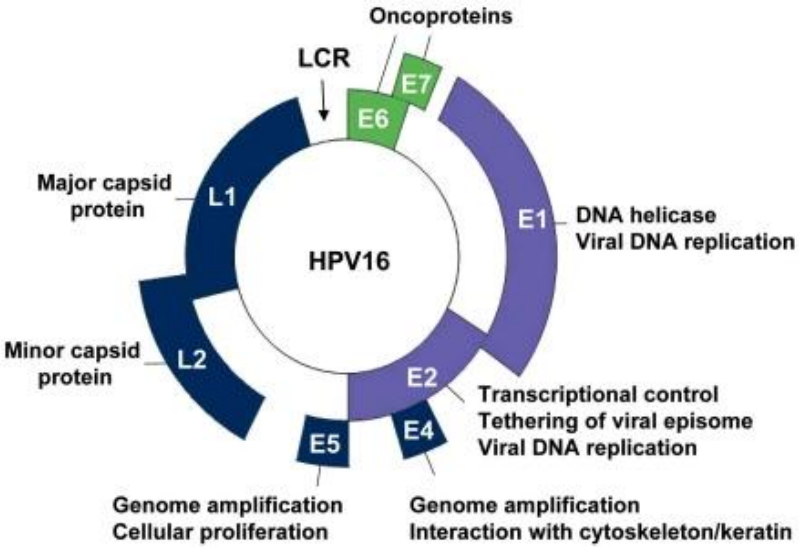


Figure 3: HPV16 genome organitation (C.M. D'Abramo and J. Archambault, 2011)

E region: early prtoiens

E1: it is involved in DNA replication and contributes to the maintenance of the genome in episomal form.

E2: it regulates DNA replication and viral transcription through the inhibition of E6 and E7 proteins. E6 and E7 genes control is lost when the viral genome integrates into the host genome. This event lead to suppress, E6 and E7 genes inhibition, which, in turn, act on p53 and pRb, two target tumor suppressor genes.

E4: it is expressed in the late stages of viral infection and is very important in the cellular maturation and proliferation. It appears to affect the integrity of the cell cytoskeleton in human keratinocytes, determining the koilocytosis, which consists in the deformation of the infected cells and subsequent leakage of newly formed viral particles.

E5: it fits into the infected cell membrane, altering the activity of membrane proteins involved in cell proliferation signals (EGF, PDGF); it also downregulates the major histocompatibility complex molecules of class I (MHC-I) and inhibits apoptosis.

E6: this gene degrades p53 by interfering with both its transcription and translation signals and apoptosis signals. The interaction with IRF-1 (Interferon Regulatory Factor 1) blocks the genes coding for immune proteins transcription induced by IFN and contributes to immune evasion. The site of integration is comprised between the 3' of E1 and 5' of E2, resulting in breaking or inactivation of E2. The functional loss of E2 results in increased expression of E6. E6 interferes with the function of p53 leading to its ubiquitin-dependent degradation that is mediated by the

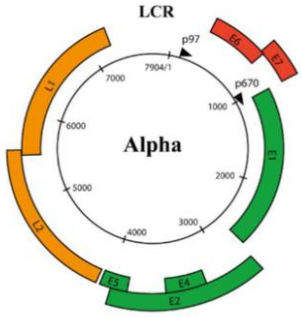
ubiquitin-ligase E6-AP. The functional loss of p53 leads to inactivation of one of the most important control systems to avoid genomic instability and the abnormal cell growth.

E7: it binds the retinoblastoma protein (pRb), which is involved in the regulation of cell growth, in cell cycle block and in repression of genes transcription involved in apoptosis. The interaction of E7 with pRb determines the release of E2F1 protein that, in the absence of E7, is linked to pRb hypophosphorylated form. E2F1, can function as transcriptional activator of cellular genes involved in the synthesis of cellular DNA and progression in S phase of the cell cycle. In addition, interaction with ISGF-3 (Interferon Stimulated Gene Factor 3) and IRF-1 (Interferon Regulatory Factor 1) contributes to the innate immune evasion of the virus.

L region: late proteins

L1 and **L2** are proteins translated late and are only expressed in productively infected cells. They will spontaneously assemble to form the icosahedral capsid. The capsid is composed of 72 capsomeres. Each capsomere is constituted by a L1 (55 kD pentamer), the major capsid protein, that constitutes 80% of the total viral protein. L1 is mainly involved in the binding to the viral DNA, but may not incorporate the DNA. It is responsible for the attack of the virus to susceptible cells and also mediates the humoral and cell-mediated infection. The capsid of each virion also contains about 12 copies of L2 (70 kD), the minor capsid protein (Burd EM, 2003). L2 mainly plays a role in structural type, but also in several regulatory functions during the life cycle of HPV, among which the bind to the secondary receptors, the determination of the virus nuclear localization and the selective DNA encapsidation into the viral capsid, thus increases the virions infectivity (Figure 4).

A



B

Core Proteins	
E1	ATP-dependent helicase. Role in papillomavirus genome replication.
E2	Coactivator of viral genome replication through the recruitment of E1 to the viral replication origin. Transcription factor of E6 and E7, also important for viral genome segregation.
L1	Major capsid protein. Assembles into pentomeric capsomeres, which are the primary components of the icosahedral virion shell.
L2	Minor capsid protein. Involved in encapsidation of viral DNA. Facilitate virus entry and trafficikng to the nucleus.
E4	E4 gene is embedded within the E2 gene, and is expressed abundantly as an E1^E4 fusion protein during the late stages of the viral life cycle. Binds to cytokeratin filaments and disrupts their structure. E4 is thought also to contribute to virus release and transmission.
Accessory Proteins	
E5	Small transmembrane protein. In Alpha-PVs, E5 interacts with the EGF receptor and activates mitogenic signaling pathways. Has a role in evading the immune response and apoptosis. Beta-, Gamma- and Mu-PVs lack E5 gene.
E6	Drives cell cycle entry to allow genome amplification in the upper epithelial layers. E6 of high-risk Alpha-PV types binds and degrades p53 and can also activate telomerase and contribute to transformation. Also involved in immune evasion. Some Gamma-PVs lack E6 gene.
E7	Drives cell cycle entry to allow genome amplification in the upper epithelial layers. E7 of high-risk Alpha-PV types binds and degrades pRb and can induces chromosomal instability. E7 is necessary for cell transformation. Both E6 and E7 have a large number of cellular substrates, with the identity of these substrates differing between HPV types [78].

Figure 4: Function of E and L regions (Egawa et al 2015)

2.3 HPV REPLICATIVE CYCLE

The HPV infection takes place through the initial contact of the virus with the surface layer of the squamous epithelium multilayered. HPV not encodes a DNA polymerase, but uses a DNA polymerase of the cellular host. For this reason, HPV replicates only in the differentiation cells. Viral DNA synthesis, capsid proteins production and virions assembly, take place only at the level of keratinocytes differentiation terminal process.

The epidermis is made up of 5 different layers: basal, spinosum, granulosum, lucidum and corneum. Corneum layer represent a terminal phase of keratinocyte differentiation, corresponding to a specific stage of the viral life cycle. Infection occurs when the virus reaches the basal layer. This is made possible by small wounds or abrasions of the host mucosa. The receptors used by the virus to make contact with the cells and penetrate inside them have not yet been clearly. It has been difficult to study the mechanisms by which HPV enters into the cells and establishes a productive infection, because of the difficulty in the production of virus both in vivo and in vitro systems. The interaction between HPV and cell surface was therefore investigated using the pseudo-virions formed by proteins L1 and L2 self-assemble in heterologous expression systems (VLP: virus-like particles) and a reporter plasmid in place of the genome (Touze A et al., 98).

HPV can bind not only in squamous epithelial cells, but also to other cell types. This indicates that the HPV marked tropism for keratinocytes is not due to the receptor specificity (Müller M et al., 1995). Integrin α6 was the first candidate as a possible receptor HPV, according to studies using the VLP (Evander M et al., 1997). VLPs bind to integrin and the use of antibodies directed against α6 blocks the binding of the virus to the cell. Integrin α6 cooperates with β1 and β4 integrin β subunit, located on the cell surface. The α6β1 integrin is expressed on a wide variety of cells, including platelets, lymphocytes, endothelial cells, while α6β4 is located on epithelial, mesenchymal and neuronal cells. HPV can bind to both integrin types, but preferentially α6β4 profile (Brendle SA et al., 2015). These integrins expression is not however necessary for entry of the virus into the cells, because some HPV enter into cells lacking these receptors. It has been observed that these viruses, bind to heparin and glycosaminoglycans, followed by subsequent receptor binding and internalization (Joyce JC et al., 1999; Shafti-Keramat S et al., 2003).

After receptors binding, the virions enter slowly into the cytoplasmic compartment (Culp TD et al., 2004), disassemble in lysosomes and then the viral DNA is transferred and transported by the minor capsid protein L2 into the core (Day PM et al., 2003).

The replicative cycle of the virus can be divided in a non-productive or early stage and a productive, or late stage, related to the state of host cell differentiation.

In the non-productive stage, HPV DNA is maintained in episomal form and early promoter begins to express E1 and E2 proteins that trigger the replication of the viral genome.

In infected basal cells, the DNA is maintained in a low number of episomal copies, about 10-100 genomes for cell. In this stage, the number of genomic copies remains constant and viral replication takes place simultaneously with host cell: the virus uses the structures and the host cellular components for the replication of its DNA.

This type of replication (replication plasmid) allows the virus to establish persistent infection in the basal cells of the epidermis, thus inducing a latent infection. A group of these cells leaves the basal membrane to stratify and differentiate, making possible the entry of the virus in the epidermis upper layers.

At this level begins the productive stage of the HPV replication cycle that affects the cells of the epithelium upper layers (granular layer). These cells are in an advanced differentiation state and there isn't the cellular DNA synthesis, but an intense viral DNA replication is present (vegetative replication), with subsequent loss of the genomic copies number control, up to thousands copies for cell. It takes place also the late viral gene expression, the capsid proteins synthesis of and virions assembly. The complete virus formation will have in the outermost epithelium layer (stratum corneum), and the viruses assembled are expelled into the outside environment, when the epithelial cells exfoliate. The virions released outside start a new infection (Figure 5).

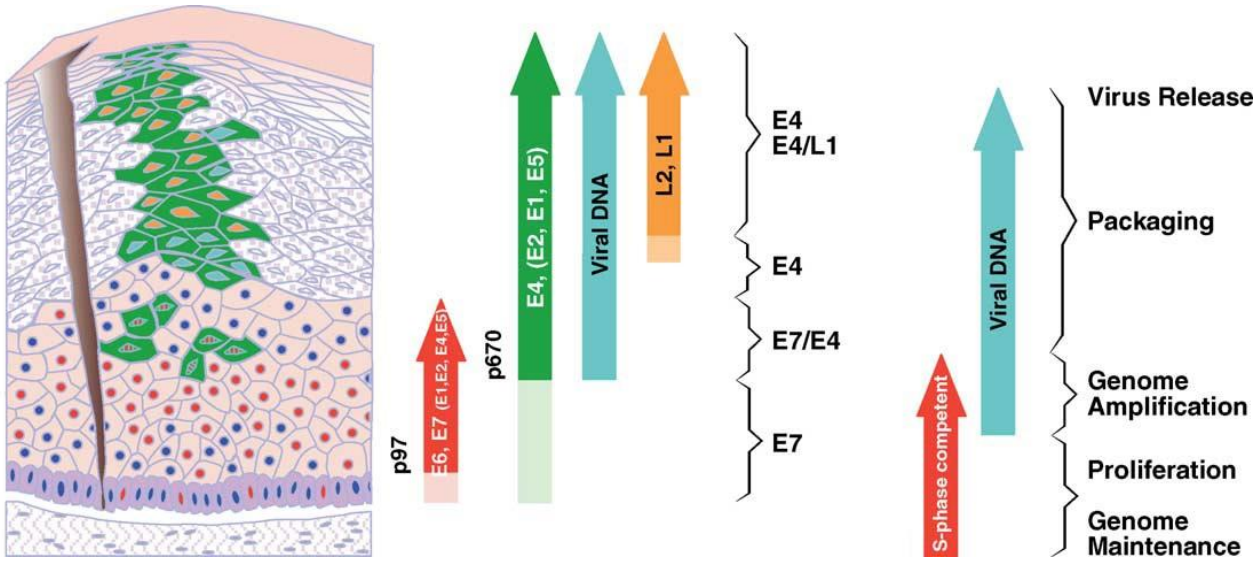


Figure 5: Life cycle organitation during HPV productive infection (Doorbar J, 2005)

2.4 HPV AND TRANSFORMATION

HPV is able to generate two types of lesions, high and low. This difference is due to the different behavior of the viral genome within the infected cell. The genome of HPV can exist in two states in the cellular host: episomal form or integrated into the genome.

The episomal form is associated with low-grade lesions or cervical intraepithelial neoplasia (CIN) and it is associated with the lag phase. The retention of this form is a critical phase of the HPV life cycle. The integrated form is highly associated with high-grade lesions and invasive cervical carcinoma. It can be integrated in single or multiple copy repeated in tandem.

The integration appears to occur in fragile sites of human genome, the regions subjected to genomic rearrangements and deletions or in the regions directly or indirectly involved in the regulation of cell proliferation. To occur integration, the viral genome must go to breaking and then to linearization. In this form, there is an interruption of the virus replicative cycle, with subsequent destruction of viral genome and cellular genes alterations. There is a loss of the region between E1 and E2 genes with a consequent non-transcription of the late genes. It is also present an uncontrolled transcription of E6 and E7 early genes, which encode for important oncoproteins involved in viral replication followed by cellular transformation and immortalization. In tumors induced by high-risk HPV genotypes, E1, E6 and E7 genes are integrated and functional, inducing cellular proliferation, while the E2, E4 and E5 genes are lost or are not transcripted.

2.5 HPV IMMUNE RESPONSE

The host cell reacts at the entrance of the virus through cooperation between the innate and the adaptive immune response. The innate immune response, does not have the characteristic of memory but it acts as soon as it notices the presence of pathogens. Its role is crucial for the adaptive immune system response, which acts both through more specific response and storing the pathogen. The antibodies produced by this system, fight against virus infected cells and allow to prevent reinfection.

The innate immune response is the first system of control, in which are products neutrophils, macrophages and lymphocytes subsequently. These agents are activated when the virus enter into the cells, due to uptake by toll-like receptor. They detect the presence of L1 and L2 capsid proteins and the HPV double-strand DNA. The innate defense system, is activated with the production of

various inflammatory cytokines, such as IL-1 beta, IL-6, IL-8, IL-12 and interferon alpha, beta and gamma, which in turn activate natural killer cells and other immune system cells (Woodworth, 2002). The second phase of immune system, consists in the adaptive immune response. In this phase, the antigen-presenting cells (APC), such as Langerhans cells or dendritic cells (DC), process the viral antigens into small peptides that are presented together with MHC to lymphocytes. DCs migrate into the lymph nodes, where happens the maturation process. The complex of viral antigen / MHC present on DCs cells, binds to specific cell receptors CD4 + and CD8 +. This binding induces T cells proliferation and activation. The activated CD4 + cells can differentiate into Th1, Th2 or Treg / Th3, that produce different cytokines: Th1 cells produce IL2, IL12, IL15, and TNFalpha; Th2 cells produce IL4, IL5, IL6, IL10 and IL13; Th3 cells produce IL10, TGFbeta and INFgamma. (Rosales R et al., 2014). The activated CD8 + cells can differentiate into cytotoxic T lymphocytes (CTL) that produce different types of enzymes. In addition, the CTLs can migrate to the site of infection and destroy cells infected by HPV. The adaptive immune response is therefore very important and effective in most cases. It includes two types of responses: humoral and cell-mediated (Figure 6).

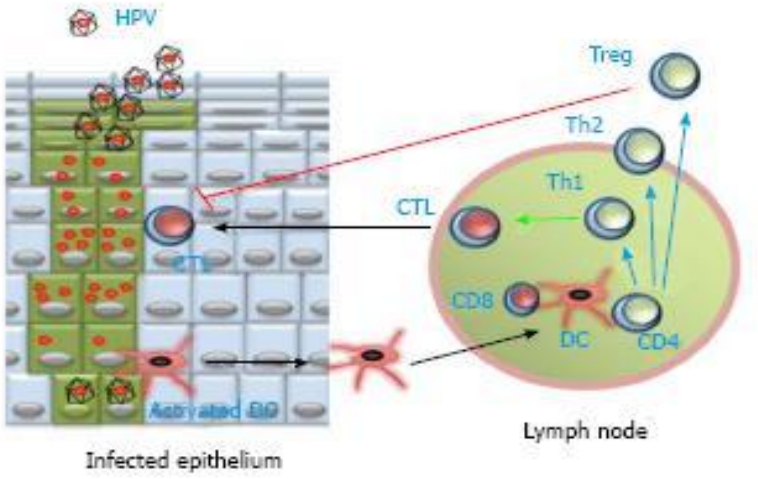


Figure 6: Cellular immune response against HPV (Rosales R, 2014)

Humoral Response

The humoral immune response is more prevalent in patients with an HPV infection, and you can detect it with the presence of antibodies against the L1 and L2 capsid proteins and E4, especially in the early stages infection. Antibodies against E6 and E7 can be identified in the later stages, when the virus is integrated into the cellular genome, especially in low and high-risk lesions. The mature B cells release antigen-specific antibodies, in order to block the virus entry and to prevent new

infections. The circulating antibody, can opsonize and agglutinate the viral particles and facilitate phagocytosis by macrophages. The antibody response is often weak and variable and does not seem to be protective for future infections. The humoral response, does not seem to be efficient to eliminate the persistent infection of HPV. In any case, the antibodies remain for a long time, even when the virus has been eradicated, so their measurement can be used to evaluate a past infection.

Cell-mediated response

The cell-mediated response is most effective against the HPV lesions. The T CD4 + and CD8 + lymphocytes are produced to eliminate infected cells. Patients that have eliminated HPV infections, have high levels of viral proteins, CD4 and CD8 circulating. A high ratio of CD4 and CD8 with a predominance of Th1 cytokines are observed in the lesion regression phase. Conversely, a low ratio of CD4 and CD8 and an increase in Th2 cytokines are observed and associated with persistent injuries. The cell-mediated response is very important for the elimination of HPV lesions.

2.6 ESCAPE THE IMMUNE RESPONSE

HPV infections are not easily recognized by immune system. The HPV, especially high risk genotype, have developed systems to evade the immune response of the target cell. HPV is not a cytopathic virus, but is an epithelium-tropic virus, that infects the basal cells of the epidermis and thanks to the differentiation of keratinocytes, the HPV infected cells arrive to the epithelial surface and become infectious.

This system lead to a viraemic phase lack and inflammation and an absence of pro-inflammatory cytokines production. The proteins oncogene E6 and E7, are able to downregulate the interferon pathway, through the inactivation of the INF alpha genes (Zivadinović R et al., 2014). E7 protein, increases the IL-18BP production, an anti-inflammatory cytokine, which mediates the activation of CD4 + T cells, causing their reduction (Richards KH et al., 2014). Finally, HPV reduces and alters the signals that lead to dendritic cells activation and migration, the MHC- I migration and APC.

2.7 VACCINES

Prophylactic vaccination anti-HPV

In the last few years, vaccines have been developed for certain genotypes. In particular, HPV16 and HPV 18 infections in high-grade lesions and frequent HPV6 and HPV11 detection in genital warts, have led to the formulation of two kinds of vaccines, both derived from virus-like particles (VLP) produced by recombinant technology. They do not contain any living biological product or DNA, and they are not infectious viral particles (Cutts FT et al., 2007). They have the ability to induce a protective immune response specific type. The bivalent vaccine, Cervarix (GlaxoSmithKline), contains VLPs for HPV16 and HPV18 types (total associated with about 70% of all cervical carcinomas). The quadrivalent vaccine, Gardasil® (Sanofi Pasteur MSD), contains VLPs which prevents lesions caused by four types of the virus: HPV 6 and HPV 11 (responsible for 90% of genital warts), in addition to HPV 16 and HPV 18 (formerly treated by bivalent).

Italy was the first European country to schedule a public vaccination strategy against the HPV virus, in particular the Agency for Drug Agency (AIFA) and the National Health Council saw fit to free vaccinate all girls with eleventh year of age (http://www.salute.gov.it).

It has been shown that 99% of vaccinated women developed antibodies and higher antibody levels are achieved in girls vaccinated between 9 and 13 years compared to those vaccinated between 16 and 26 years (Italian journal of public health, QIJPH-2014).

The efficacy of the quadrivalent vaccine to preventing cervical cancer and warts, evaluated on a population of 18.000 women, was 95-100% (FUTURE II Study Group., 2007). The effectiveness of the divalent form is around 90% (Cutts FT et al., 2007). This type of strategy, targeted to vaccination of not sexually active adolescents that do not come into contact with the virus, it can be definitely an advantage for prevention; In this regard it is evaluating the effectiveness of the vaccine also of sexually active adolescents (Villa LL, 2007).

A second generation of anti-HPV vaccine was approved by the FDA in December 2014. It is a ninth-valent vaccine (HPV9) that provides protection for the same types of HPV4 (quadrivalent) with the addition of five HPV genotypes (31, 33, 45, 52 and 58). HPV9 provides protection against the 80% -90% of cervical tumors, about 10% -20% more than the vaccines approved previously and has been shown to be safe and effective (Fontenot HB et al., 2015).

Therapeutic vaccins

During these last years, it is developing a therapeutic vaccine type targeted to induction of a cell-mediated immune response specific to particular tumor antigens, such as E2, E6 or E7.
This type of vaccine would be aimed to all women who have a persistent infection and are generally not helped by a prophylactic vaccine, to the men and women susceptible to HPV infections in other locations, including oral cavity and the anus and towards countries where it is still high mortality from cervical cancer. At present, are under study five different methodologies, based on the administration of antigenic peptides or recombinant proteins, viral vectors and presenting dendritic cells as antigen E7 or E6. The results of clinical trials are not yet clear, but in some cases seem to give good results (Nonn M et al., 2003; Ferrara A et al., 2003).

2.8 CLINICAL MANIFESTATION FROM HPV

HPV may give to different types of clinical manifestations, depending on the affected anatomical region. HPV can infect skin, generating both benign events, with formations of warts on the back of the hand, feet or face, and malignant events, such as the onset of skin cancers. A mucosal level, HPV infection may give rise benign manifestations, with the formation of male and female genitalia warts and lesions in aerodigestive tract. Especially, the uterine cervix, respiratory and anogenital tract are subjected to a mucosal malignant transformation.

2.8.1 Hpv and Breast Cancer

Over the past 25 years, several studies have attempted to relate HPV with breast cancer. In 1990, it was described and demonstrated that HPV can immortalize human mammary epithelial cells (Band V et al., 1990). In 1992, a study described the presence of HPV16 in 29% of breast tumors and metastatic lymph nodes (Di Leonardo A et al., 1992) and later was described a relationship between breast and cervical cancer tissues, where it was identified HPV16 (Hennig EM et al., 1999).
In patients with breast cancer, HPV has been associated with very different populations, especially the 16, 18 and 33 high-risk genotypes (Amarante MK et al., 2009).
Identify HPV in breast cancer is very difficult, due to the low viral load of virus present in breast tissue (Herrera-Romano L et al., 2012), and the low-sensitive techniques to detect it. Despite these difficulties, the HPV sequences have now been identified in mammary tumors in several studies.

Recently meta-analysis study was conducted on 22 case-control studies. 2845 samples were analyzed, including 1897 cases of breast cancer and 948 controls. The results indicate that HPV infection is associated with an increased risk of developing breast cancer, compared to controls (odds ratio 4.2) (Bae JM et al., 2016).

It has been shown that the prevalence of HPV high-risk is high in early breast cancer as compared to late breast cancer, and it is due to the HPV action on the APOBEC3B pathway (Ohba et al., 2014; Vieira VC et al., 2014).

Finally, it was observed that women with an HPV positive cervical disease, can develop, HPV positive breast cancers and these women are subject to a greater risk of developing this type of tumor (Lawson JC et al., 2016).

The role of HPV in breast cancer, appears to be of great interest. On the basis of the data reported in literature, it seems that it can influence the development of cancer, increasing the risk of occurrence, but it remains to clarify the way through which it reaches the breast tissue.

2.8.2 Hpv and uterine cervix

It was observed and described that some HPV genotypes, are associated with the onset of the uterine cervix cancer and are particularly interested squamous cells (80 -90%) (Burd EM 2003). Usually, when the genital infection is subclinical, it doesn't cause injury but it resolves spontaneously (SIL). Otherwise, the infection can progress and generate low-grade intraepithelial lesions (LSIL). The LSIL may regress spontaneously or persist and progress to squamous intraepithelial lesions of high grade (HSIL) and carcinoma in situ. It is possible to classify cervical cancer at histopathological level, depending on the change and degree of injury: CIN1, is characterized by episomal viral DNA form and by E6 and E7 expression gene relatively low (Schiller JT et al., 2010); CIN2, is characterized by the presence of both episomal and integrated viral DNA form. In this phase, cells that have integrated viral DNA, overexpress the viral proteins, which lead to an increase of cell proliferation and degree of malignancy. At this point the lesion evolves towards CIN3, characterized by keratinocytes which lost the ability to regulate the proliferation and cell differentiation, and gained the ability to uncontrollably express E6 and E7 oncoproteins. (Stanley M, 2003). The only infection from HPV high risk is necessary, but not sufficient to trigger the tumor progression. Other host and environmental factors, including genetic and epigenetic events are required to induce tumor progression. (Zur Hausen H, 2002).

2.8.3 Hpv and oral cancer

Although the majority of cervical cancers are caused by HPV, there is a small portion of the non-cervical tumors, including oral cancer, which undergo to HPV infection (Kim SM, 2016). Oral cancer is classified as head and neck cancers (HNSCC). Unlike HNSCC, in which the causes appear to be due primarily to alcohol and smoking, recently, oral cancer has been associated with HPV infection, in particular the 16 genotype. (Sun JR, 2012; Gillison ML et al., 2008). There was a significant association between HPV infection, oral squamous cell carcinoma and, although variably, malignant oral cavity lesions, such as leukoplakia and erythrocyte erythroplakia. (Pierangeli A et al., 2016). This type of cancer, occurs mainly to tonsillar level, on the base of tongue and on the soft palate and mostly affects people under 50 years old. This disease is transmitted sexually and by skin contact, so, the frequent change of partners without using precautions, oral sex with people with HPV positive genitals infections and the first sexual experience at an early aged, can be factors predisposing to the onset of HPV infection and tumor (Heck JE et al., 2010).

2.8.4 Hpv and colon

The pathology of colorectal cancer is considered a multifactorial disease: the carcinogenesis model, shows a progressive accumulation of genetic damage, which can lead to polyps formation and subsequent evolution in cancer. A small portion of cases is characterized by genetic predispositions, while in the others cases are well described the external factors, such as lifestyle, a sedentary lifestyle, abuse of alcohol and red meat, a high fat-diet and low fiber-diet or biological agents, although the molecular mechanisms are not yet completely understood (Perez LO et al., 2010; Beart RW et al., 1995; Gazelle GS et al., 2000; Vainio H et al., 2003).

Kirgan et al (1990), described a potential association between HPV and colorectal cancer. Recently, several studies have been reported, HPV and the risk of tumor (Yavuzer D et al., 2011; Lorenzon L et al.,2011; Giuliani L et al., 2008). HPV infection in colorectal cancer could be due to an infection coming from anogenital sites (Perez LO et al., 2005) or through blood and lymphatic circulation (Chen TH et al., 2012; Chen AC et al., 2009). Other studies report an association with sexual and behavioral habits (Veldhuijzen NJ et al., 2010). However, the meta-analysis studies conducted in recent years (Damin DC et al., 2013; Baandrup L et al., 2014; Pelizzer T et al., 2016), suggests an important role of HPV in colorectal cancer, since it is considered a possible co-factor for the development of diseases.

3. POLYOMAVIRUS

The polyomavirus family is composed by a DNA non-enveloped viruses of about 5000 bp, that infect a large number of vertebrates, including humans, primates, mice, rabbits, and many species of birds. (Imperiale MJ, 2001; Delbue S et al., 2012). Capsid is composed by three viral proteins designated VP1, VP2 and VP3. Viral genome has a similar organization between the various virus and presents highly conserved regions. The first human polyomavirus, were identified in the 1970 and their names are derived from the initials of the subjects in which they were identified: JC (JCV) was found in patient brain with Hodgkin's disease and progressive multifocal leukoencephalopathy (PML) (Padgett BL et al., 1971); BK (BKV) was found in the urine of a renal transplant (Gardner SD et al., 1971). In recent years were found and described other viruses: in 2007 were found the polyomavirus KI (Karolinska Institutet) and WU (Washington University) in airway samples from patients with lung disease, also known by the initials of patients who were identified (Allander T et al., 2007; Gaynor AM et al., 2007); in 2008 the Merkel Cell virus, was found in > 80% of the samples of Merkel cell carcinoma, rare neuroendocrine tumor of the skin, but also in samples of numerous other tissues from healthy individuals affected by different pathologies (Feng H et al., 2008); in 2010 the 5 and 6 polyomavirus (PyV5 and PyV6) were detected in skin samples (Schowalter RM et al., 2010) and Trichodysplasia spinulosa virus (TSV) was associated to the homonym and follicular rare inflammatory skin disease seen in patients deeply immunocompromised such as organ transplant recipients (van der Meijden E et al., 2010) . In human samples there were also detected even primate polyomavirus: in particular, Simian Virus 40 (SV40) was introduced in humans with the use of anti-polio vaccines in the 60s prepared on cell cultures of infected monkey; the Lymphoptropic Polyomavirus, for which it seems a relatively high frequency (15%) of exposure on the basis of epidemiological data serum (Kean JM et al., 2009). SV40 was also found in people who do not was administered polio vaccine, so this leads to the assumption that the propagation has happened not only by immunization, but also to vertical and / or horizontal transmission (Shah KV, 1972). It was also associated with human neoplastic diseases, but without some evidence because frequent plasmid detectable contamination in laboratories around the world. Although the sequences of Polyomavirus are highly conserved, they have a restricted host range: they productively infect permissive cells and lead to neoplastic transformation of non-permissive cells, generating different malignancies. The oncogenic transformation is mediated by early proteins, called antigens T, who manage to cross the cell cycle controls and

replicate efficiently. The Polyomavirus establish a primary infection, typically latent, which tends to be associated with a symptomatic clinical picture only under certain conditions.

3.1 POLYOMAVIRUS STRUCTURE

The polyomavirus are small circular double-stranded DNA viruses of about 5000 bp, without enveloped, with an icosahedral capsid of 45 nm. The capsids are composed of three viral proteins (VP1, VP2, VP3) that surrounding the DNA assembled in the form of chromatin with H2A, H2B, H3 and H4, cellular histones. This complex is often called minichromosome. The capsid proteins are organized to form an icosahedral capsid of 360 molecules of the major capsid protein VP1, organized in 72 pentameric capsomeres. The VP2 and VP3 protein (minor capsid protein) are associated with each pentamer.

3.2 POLYOMAVIRUS GENOME

The genome of all polyomavirus is about 5000 bp and is divided into three regions: precocious (early), late (late), and regulatory expertise. (Cole and Cozen, 2001). In the early region are expressed a set of multifunctional regulatory proteins, tumor antigens (T / t), immediately after the entry of the virus into the cell and continue to be expressed, after infection and the onset of viral replication. Genetic and biochemical analysis of the SV40 T antigen and MPyV revealed that the large T antigen (TAg) is essential to drive the infected cells in quiescent phase to the S phase of the cell cycle, to initiate and complete replication viral DNA and to promote the virion assembly (Ahuja D et al., 2005). In the late stage, the three capsid proteins are expressed (VP1, VP2 and VP3) and even in some cases an agnoproteina, so called because of its unknown function: it seems that facilitate nuclear localization of the VP1 protein and promotes the assembly of viral particles and the subsequent spread of the virus from cell to cell. The regulatory region (RR) is located between the early and late transcriptional unit and contains a bidirectional enhancer, promoters for the early and late region, the origin of viral DNA replication and the signal for the assembly of virion (Figure 7).

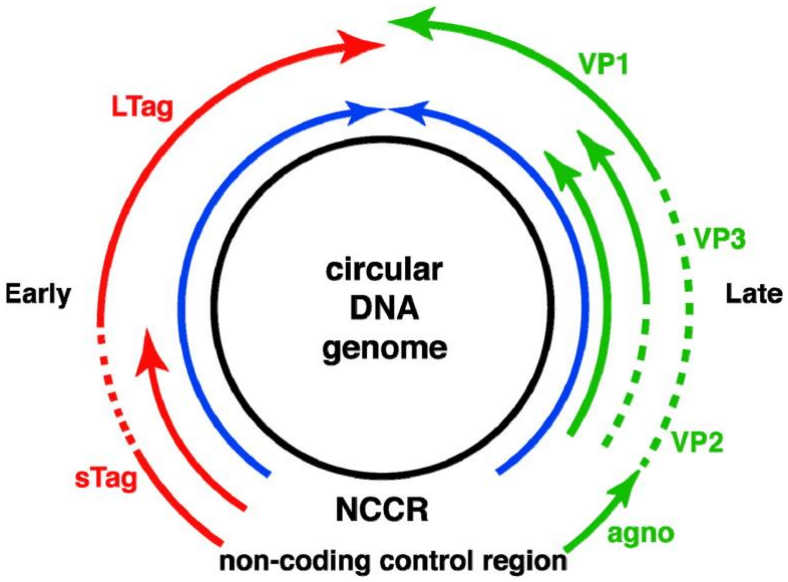


Figure 7: Scheme of a prototype PyV genome (Tina Dalianis and Hans H. Hirsch, 2013)

3.3 REPLICATION CYCLE

The viral replication begins after virus adsorption in the host cell. The virus enters into the cell and is transported directly into the nucleus, where it is released and made available to begin the transcription of the early viral genes through the host cell transcriptional apparatus operates. The primary transcript of the early region undergoes alternative splicing and leads to the formation of multifunctional regulatory proteins (small and large T). The antigens T stimulate cells to produce enzymes necessary for the replication of cellular DNA, thus preparing the cell to viral DNA replication. The late viral genes are then expressed and they code for capsid proteins. In this step, there is a new virions assembly in the nucleus of infected cells and release of the virus, probably at the time of cell death.

3.3 SV40

SV40 was isolated for the first time in 1960 from a monkey kidney to produce the polio vaccine. In animals were observed different modes of virus transmission through urine and respiratory route (Horvath CJ et al., 1992) and also were observed tumorigenic properties (Eddy BE et al., 1962; Diamandopoulos GT, 1972; Vilchez RA et al., 2003). Because of the accidental administration of the polio vaccine in humans (1955-1963), this virus has aroused particular interest to understand its functionality in tumors. DNA sequences similar to those of SV40 have been found in a wide variety

of human tumors, such as, for example, choroid plexus tumors (Lednický JA et al., 1995) medulloblastomas (Huang H et al., 1999) and in bone tumors (Carbone M et al., 1996) where SV40 DNA sequences were found in 40-50% of mesotheliomas, albeit variably in relation to geographical areas (Emri S et al., 2000). Numerous studies raise many concerns, because most of human cancers associated with SV40, correlate with the tumors observed in experimental animal models.

There are different methods to detect SV40, but the obtained results are still not clear. It remains to be seen whether the virus could significantly contribute to the onset disease or whether it can increase a compromised condition and if in some cases, the pathologic microenvironment, may provide a trigger to reactivate a latent SV40 infection (Figure 8).

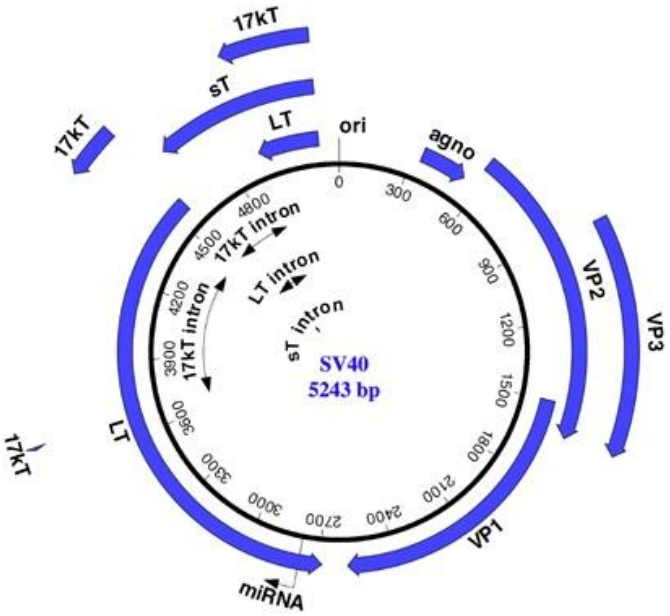


Figure 8: SV40 genomic DNA (Ahuja et al., 2005)

4. EXTRA-CELLULAR VESICLES

In recent years, it has been suggested a cell-cell communication mechanism, mediated by extracellular vesicles. The extracellular vesicles are released from different types of cells, tissue and biological fluids and poured into the extracellular environment. They are divided according to different criteria: sizes (microparticles, microvesicles, nanovescicole and nanoparticles); type of cell or tissue origin (prostasoma and oncosoma); functions (calcifying matrix vesicles, argosomes, tolerosomes) and their presence outside cells (ectosoma, exosome complex, esovescicole, exosome-like vesicles) (Colombo M et al., 2014).

Although it was already described the release of apoptotic bodies during the process of apoptosis (Hristov et al., 2014), the release of vesicles by properly functioning cells was described only in recent years. In particular, microvesicles, ectosomi, shedding vesicle and exosomes are described (Gyorgy et al., 2014). Ectosomi, shedding vesicle, microparticles and microvesicles, are contained within a range from 150-1000 nm, while the exosomes have a size between 30-150 nm. Despite differences in terms of size, composition, morphology and density, it is difficult to distinguish exosomes from the microvesicles group (Bobrie A et al., 2011).

4.1 EXOSOMES

In 1970, it was described vesicles enclosed by a membrane, made by different types of tissues, cells and biological fluids (Anderson HC 1969; Trams EG 1981; Dvorak AM 1981). In 1980, it was described a more complex pathway of microvesicles secretion, which provided for the release of vesicles formed within multivesicular endosomes (MVB) (Pan BT et al., 1985). The term "exosome" appears in the following years, when the Dott. Johnstone described them as small membrane vesicles, released from the MVB system, during the development of reticulocytes, whose job was to clean the cellular waste from the cytoplasm during reticulocytes maturation (Johnstone RM, 1987). For several years, exosomes have assumed a marginal role until the end of the 90s, when their potential importance as mediators in intracellular communication was described (Raposo G et al., 1996; Zitvogel L et al., 1998). Exosomes secreted by Epstein-Barr virus (EBV) - transformed B cell lines containing MHC II (Raposo G et al., 1996) and exosomes produced by B lymphocytes in vitro were able to stimulate the response mediated by T cells (Zitvogel L et al., 1998). The hypothesis of exosomes as mediators of the immune response and intracellular communication, led to further investigation about their functions.

In recent years, several studies describe the role of exosomes: has been observed their role in cell-cell and distance communication, interacting with the recipient cells (Lo Cicero A et al., 2015). It was highlighted their contribution in tumor progression by promoting angiogenesis and metastatic tumor cell migration (Rak J, 2010; Hood JL et al., 2011; Peinado et al., 2012); it was also noted their inactivators role of immune response (Zhang HG and Grizzle WE et al., 2011).

Exosomes are secreted in both pathological and physiological condition from numerous types of cells, such as dendritic, hematopoietic, epithelial, nervous system, fibroblasts and cancer cells. Their

production increased in stress condition, for example, temperature or ph of membrane changing, hypoxia, oxidative stress, viral infections and radiation.

Exosomes are composed of many molecules. There is a database, which describes all the microRNA, mRNA, and proteins found in these vesicles. From the analysis of 146 studies were identified and associated with the exosomes, 13333 proteins, 2375 mRNAs and 764 miRNAs (www.exocarta.org). RNA and microRNA were the first to have been found and described in exosomes (Valadi et al., 2007) and they have been studied to understand their functionality (Skog J et al., 2008). Early studies of proteomics, showed that the exosomes contain specific cellular proteins content, some of which depend on the cell type from which they come. To date the exosomes are constituted mainly by endosome, cytosol and membrane proteins, and to a lesser extent by nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus proteins (Colombo et al., 2014). It was also analyzed the lipid content, observing a prevalence of fatty acids, sphingomyelin, posphotidilcholine, ceramide and cholesterol (Laulagnier et al., 2004; Llorente et al., 2013; Trajkovic et al., 2008). The contents of exosomes are also enriched by DNA (Jin Cai 2016), viral nucleic acids (Gould et al., 2003; Laganà et al., 2013; Hoen et al., 2016), transmembrane, fusion, cytoskeleton and membrane transport proteins, enzymes, signal transduction, adhesion molecules and antigen presenting molecules (Colombo et al., 2014). How exosomes exert their functions is not yet entirely clear. (Figure 9)

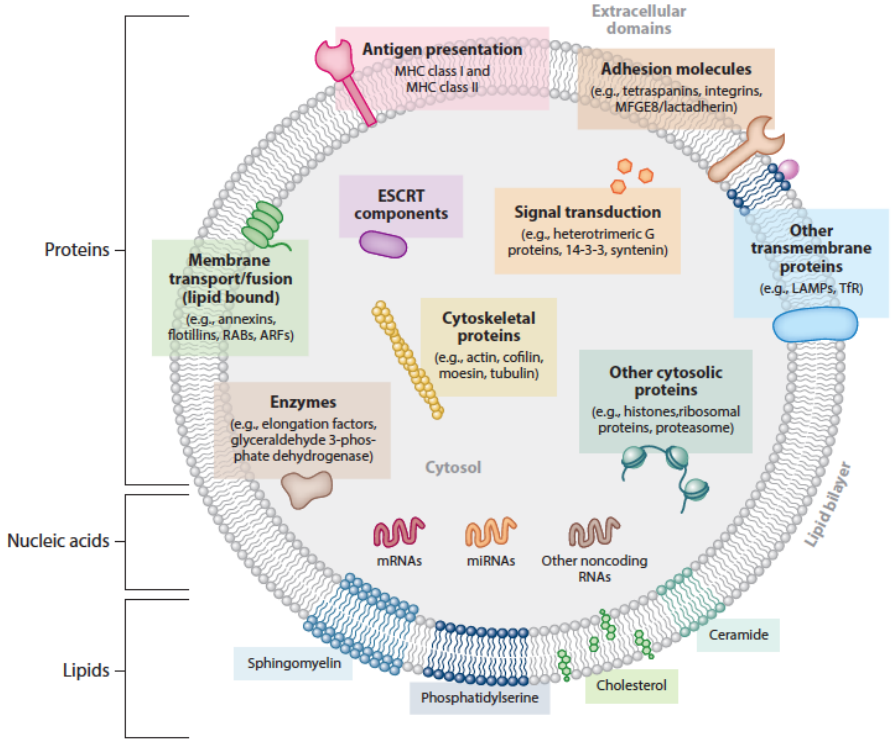


Figure 9: Schematic representation of exosomes composition (Colombo M et al.,, 2014)

4.2 BIOGENESIS OF EXOSOMES

Exosomes originate from the endosomal system, which generates the different intraluminal vesicles, that are direct in the right directions: some of them can expect degraded by the lysosomal system, while others will face recycling or exocytosis. Endosomes are divided into three compartments: early, late and recycling. Early endosomes fuse with endocytic vesicles and incorporate their content, which can be recycled, degraded or esocitated. The vesicles destined for recycling, are directed to the recycling compartment, while the remaining early endosome, with its content, undergoes a series of transformations and becomes late. During this process, the endosomal content, is divided into many small vesicles of 30-100 nm (ILVs), which fuse directly with the lumen of late endosome and are called multi-vescicular body (MVBs). Late endosomes can fuse with the lysosome resulting in destruction of the endosomal content, or can fuse with the plasma membrane, followed by vesicles secretion in the extracellular space. These vesicles are called exosomes. They can reach the target cells in different ways: they can attach and fuse with the membrane cell, releasing their contents, or can be endocitate and then fuse with the membrane of a cell compartment, pouring their material (Raposo G et al., 2014).

Exosomes can be characterized at the molecular level, thanks to the presence of certain proteins features, which appear to result from the process of ILVs formation.

The ILVs would be formed through the triggering of two different processes. In the first, the endosome membrane, forms a specialized region, rich of tetraspanine, a class of membrane proteins (Pols MS et al., 2009). The tetraspanine consist of four transmembrane domains, interconnected by variable sequences, that enhance cell-cell specific binding. CD9 and CD63 are two tetraspanine that seem to be involved in the formation of exosomes and they are used as markers for exosomes characterization and isolation (Jansen FH et al., 2009; Kosaka N et al., 2010).

The second process involves approximately thirty proteins that assemble each other and form 4 complex, called endosomal sorting complexes required for transport (ESCRT 0, I, II, III) (Wollert T et al., 2010; Hurley JH, 2010; Babst M et al., 2002). ESCRT 0 has the duty to recognize and seize the ubiquinate transmembrane proteins in the endosomal membrane; ESCRT I and II are responsible for membrane deformation into buds, while the ESCRT III, is responsible for the membrane formation and the ILVs cleavage. The recruitment of ESCRT III takes place through Alix, a protein that binds simultaneously TSG101, ESCRT I component, and CHMP4, ESCRT III component. Tsg101 and Alix are two proteins used for exosomes characterization (Wollert T et al., 2010; McCullough J et al., 2008; Akers JC et al., 2013).

The way through which the exosomes are internalized by receiving cells, has not yet been completely clarified. It is assumed that the exosomes may enter into target cell, through simple fusion with host cell membrane, releasing their contents directly into the cytoplasm; through endocytosis, or due to the presence of specific receptors that allow exosomes-recipient cell binding (Gajos-Michniewicz A et al., 2014). The cells from which exosome originate, are decisive both for exosomes superficial contents formation and to address them towards the appropriate recipient cell (Taylor DD et al.,2013). A single exosome presents different adhesion molecules on its surface and it is capable to bind different cell surface receptors (Record M et al., 2014) (Figure 10).

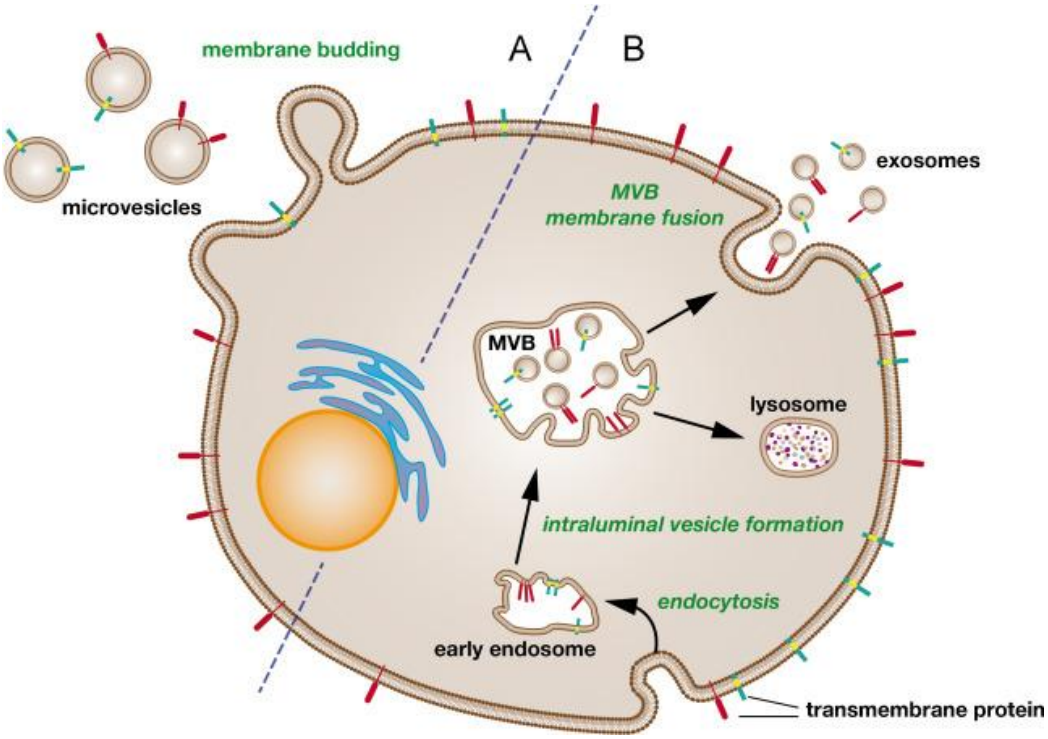


Figure 10: Formation and release of exosomes (Junker K, 2016).

4.3 EXOSOMES AND TUMORS

Tumor is a multistep process that involves several microenvironment changes, resulting in uncontrolled cell proliferation. The tumor microenvironment undergoes changes due to cancer cells genetic instability and the induction of some factors, such as cytokine and immune cells increased, inflammatory response, promotion of angiogenesis, cell migration and invasion, hypoxia environment.

Several studies describe the release of exosomes by tumor cells, which appear to be involved in normal cancer cell-to-cell communication and contribute with other factors to the progression and maturation of the tumor microenvironment. The role of exosomes has been deepened in different types of tumors (Kharaziha P et al., 2012).

Exosomes are increased in patients with cancer, due to a greater release by tumor cells, and their increased expression seems to correlate with a greater tumor size (Logozzi M et al., 2009). Exosomes are involved in cell invasiveness, since they are capable to change stromal cell, remodeling extracellular matrix and stimulating angiogenesis, especially exosomes released in hypoxic conditions (Katoh M, 2013; Khalyfa A et al., 2016). Exosomes play a role in tumor cell migration and in the acquisition of invasive properties in non-cancerous cells (Epple LM et al., 2012; Xiao et al., 2012).

The exosomes would seem to contribute to the formation of pre-metastatic niches, through the transport of biological molecules necessary to metastatic process (metalloproteases, angiogenesis factors, tetraspanin), and promoting the migration and tumor cells invasion (Fleming A et al., 2014). The formation of the pre-metastatic niches is a fundamental requirement, for tumor metastases. It was shown that melanoma metastases, uses the way of the MET receptor to modify the characteristic phenotype of progenitor bone marrow cells, in a pro-angiogenic and pro-metastatic phenotype (Peinado H et al., 2012). It has also been described that exosomes released by stromal cells of a gastrointestinal tumor, manage to convert the smooth muscle cells in a pre-metastatic sense (Atay S et al., 2014). The direct involvement of exosomes released by metastatic cancer cells was observed in vitro and in vivo through the use of imaging fluorescence methods (Suetsugu A et al., 2013).

Exosomes appear to play a very important role in the development of a tumor (Figure 11).

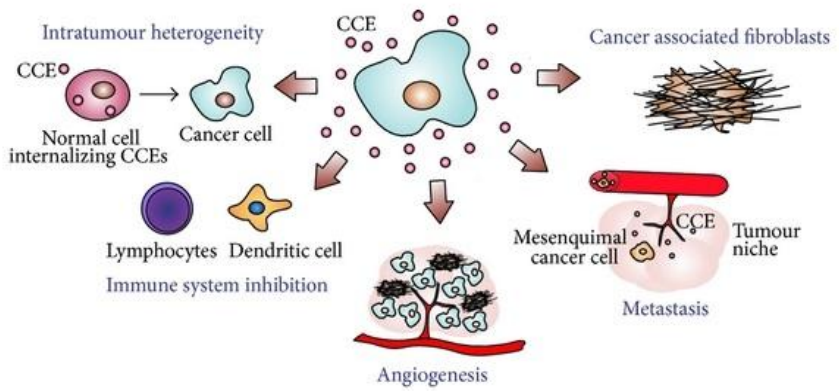


Figure 11: Role of exosomes in tumor microenviroment (Roma-Rodrigues C, 2014)

4.4 EXOSOMES AND IMMUNE SYSTEM

The exosomes produced by cancer cells, seem to be able to inhibit the immune system cells function. It has been reported that exosomes produced by human prostate cancer cells, downregulate NK and CD8 + T cells expression, block the differentiation of murine myeloid precursor cells into dendritic cells (Yu S et al., 2007) and decrease the number and function of antigen presenting cells (APC) (Lundholm L et al., 2014; Yu S et al., 2007; Berchem G et al., 2015).

On the other hand, it was also described the role of exosomes in immune response. The presence of tumor antigens, has been exploited to stimulate dendritic cells, inducing CD8 + T cell and increasing immune response (Bu N et al., 2011). Some miRNAs present in exosomes produced by cancer cells, can trigger the immune response by binding to Toll-like receptor (Fabbri M, 2012). The exosomes can be both mediators and modulators of the immune response and can play a role both of inflammatory tumor response that anti-tumor immunity (Figure 12).

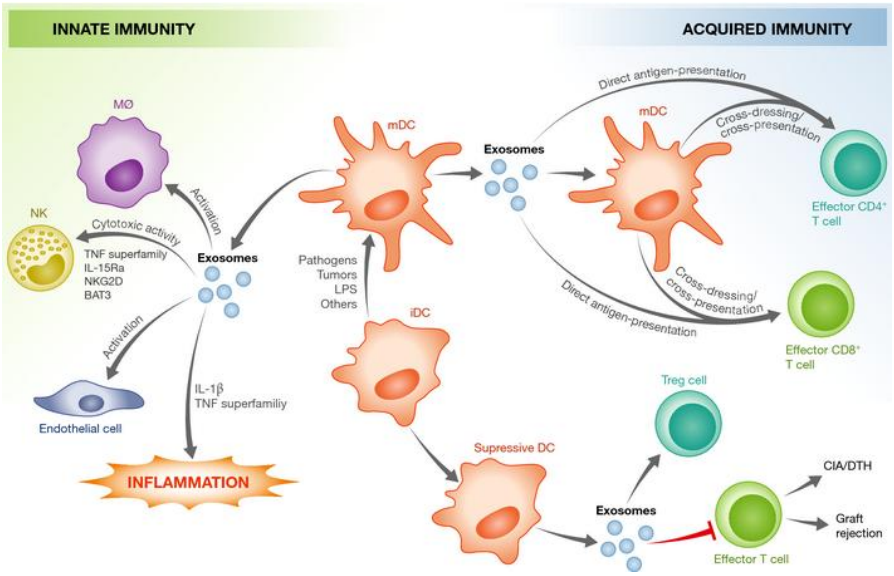


Figure 12: Role of exosomes in immune response (Schorey JS, 2014)

4.5 EXOSOMES AS BIOMARKERS AND ANTI-CANCER DRUG

Several studies have described and demonstrated the role of exosomes as a biomarker for the diagnosis of cancer. (Roma-Rodrigues C et al., 2014; Dijkstra S et al., 2014).

Each tumor is characterized by a particular protein or miRNA profile. Several studies have shown a correlation between the protein and miRNA exosomes content with the stadium and the evolution of tumor. In metastatic breast cancer cells, have been described a protein, the periostin, which has been found and validated in a cohort of patients with breast cancer metastatic lymph nodes (Vardaki I et al., 2016). A number of prostate cancer lncRNAs have been identified and 8 specific microRNAs have been validated in serum-derived exosomes from ovarian cancer patients whose expression levels were similar to those found in cancer cells (Ahadi A et al., 2016; Taylor and Gercel-Taylor, 2008). Exosomal miRNAs have been studied and identified in several types of cancer suggesting that they can play a role of probable prognostic and diagnostic indicators (Wang WT et al., 2014; Skog J et al., 2008; Rabinowits G et al., 2009; Lau C et al., 2013).

Finally, exosomes may be used to carry drugs or nano-molecules against cancer, such as miRNA and siRNA (van den Boorn JG et al., 2013; Shtam TA et al., 2013; Zhang HG et al., 2014).

The use of exosomes as drug-carriers, could bring an advantage in the fight against cancer: exosomes derived from autologous tumor cells, may induce less toxicity and immunogenicity to the recipient cells, compared to artificial drugs administration; they can fuse directly with the plasma membrane, improving drug cellular internalization; finally, their diffusion in the tumor tissues would be supported both by their relative stability in biological fluids and by their small size that would allow to evade phagocytosis by macrophages (Kosaka N, et al., 2012; Ohno S, et al., 2013; Tian Y, et al., 2014; Jang SC, et al., 2013).

4.5 EXOSOMES AND VIRUS

The role of exosomes as viral acid nucleic cargo, has been described initially for the Epstein-Barr virus (EBV) and the human immunodeficiency virus (HIV). EBV was the first virus in which were found microRNAs (Cai X et al., 2006; Pegtel DM et al., 2010) demonstrated their functionality in vitro, transferring exosomes in non-infected cells, through the use of cocultures. In 2000, it was shown that the CCR5, HIV coreceptor necessary for viral entry into the cell, can be secreted from

CCR5 + cells, in vesicles form, and be transferred to CCR5- cells, demonstrating its capabilities (Mack M et al 2000).

In recent years, the release of viral nucleic acids exosomes content to recipient cells has been described in vitro for other viruses, including hepatitis C virus (HCV), hepatitis A virus (HAV) and herpesvirus 6 (HHV-6), (Greenhill C, 2013; Feng Z et al., 2013; Ota M et al., 2014). In 2012, Gaiffe E et al., described the presence of HPV DNA in cultures of fibroblasts, following exposure with apoptotic bodies containing HPV, obtained from HeLa and Caski cell lines (Gaiffe E et al., 2012). An alternative infection mechanism was described for HIV. This mechanism, called trans-infection, used the uninfected dendritic cell-to capture HIV-1 and mediate viral transfer to T cells (Izquierdo-Useros N et al., 2012).

The cargo properties of exosomes, was exploited by viruses to carry and release their viral genome into recipient cells and to spread infection. Exosomes enter into receiving cells through binding to receptors present on the surface of target cell, or fusing directly with plasma membrane of the cell, releasing their contents directly into the cytoplasm (Izquierdo-Useros et al., 2011). This system could allow the virus to evade the immune system and promote infection (Gould SJ et al., 2003).

The mechanisms related to viral transfer, are still unclear, as it is difficult to distinguish exosomes from the viral particles. For this reason, some methods have been described which allow to separate the virus from exosomes, to understand the virus-exosome complex dynamics (Nolte-'t Hoen E et al., 2016; Schwab A et al., 2015).

The role of exosomes in viral pathogenesis, could be exploited in the future for new vaccines or antiviral therapies development (Figure 13).

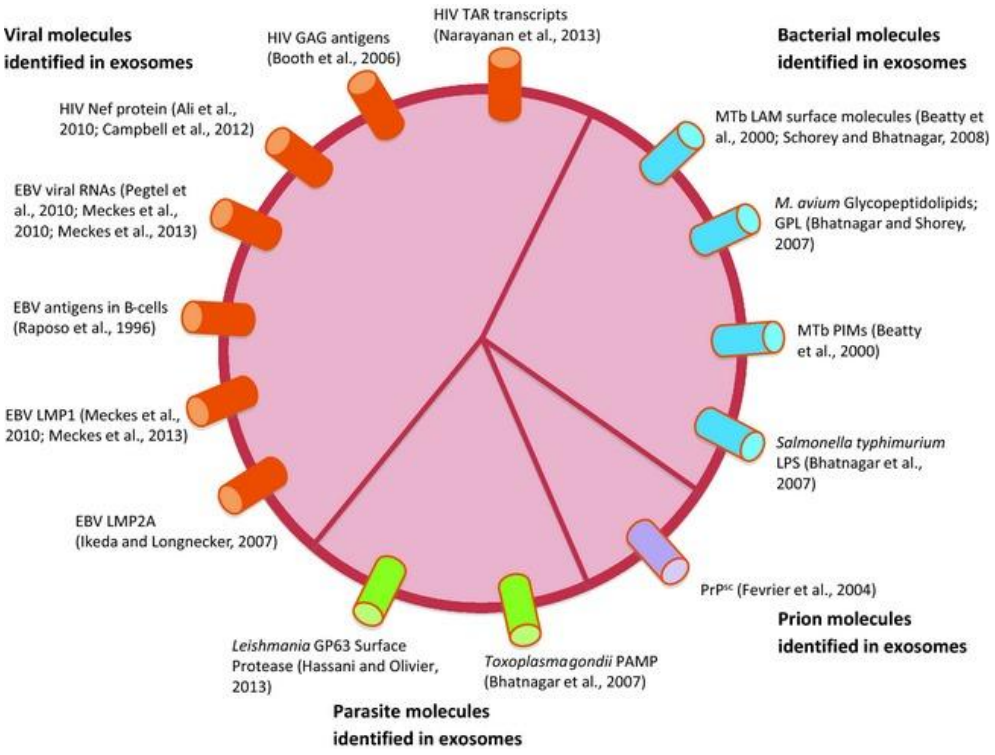


Figure 13: Pathogen-derived molecules identified in exosomes (Fleming A et al., 2014)

2. BACKGROUND AND AIM OF THE STUDY

Human papillomavirus (HPV) is one of the most frequently sexually transmitted agent and play a causative role in almost all cervical cancers in which HPV DNA is frequently found integrated into human genome at fragile sites. HPV DNA has been found also in a series of extra-genital tissues such as oro-pharingeal, skin, lung and colon tissues, in which the virus is alleged to exert an oncogenic activity. Recently HPV DNA was found to be significantly associated with breast cancer in meta-analytic studies (Simões PW et al., 2012; Bae JM, 2016). Despite this finding the role of HPV in breast cancer pathogenesis is still a conundrum. Moreover there are no clues about the route of HPV dissemination to the mammary gland tissue.

In this regard, despite the absence of overt viremia, some authors speculate the diffusion of HPV from the genital site of infection to other districts, by blood (Hennig EM et al., 1999; Widschwendter A et al., 2004). Accordingly, the presence of HPV DNA in blood samples has been detected in PBMCs and plasma in patients affected by HPV positive cancers (Bodaghi S et al., 2005).

The term microvesicles (MVs) currently includes exosomes, apoptotic bodies and shedding microvesicles, which are found in many biological fluids (blood, urine, saliva and milk) and are grouped on the basis of biophysical properties (Bobrie A et al., 2013). The process of tumorigenesis leads to an increase in exosomes secretion, whose functional properties include modulation of angiogenesis, cell invasion, but also the activity as cargo for nucleic acids (Rak J, 2010). Indeed, exosomes associates with the transmission of certain pathogens (e.g. HIV) and oncogenes which may promote the development of tumors at distant sites as a result of horizontal gene transfer.

Our preliminary data confirm the presence of HPV DNA in breast cancer tissues and serum. Intriguingly, in situ hybridization analysis allowed us to localize the DNA into epithelial and stromal compartments of the mammary gland.

In this study we plan to verify the hypothesis that HPV DNA/RNA takes part to extra-genitals and breast cancers pathogenesis. In particular, we aim to shed light on the mechanisms responsible of HPV DNA spreading from the primary and canonical site of infection, i.e. genital organs, to the others compartments by collecting exosomes through differential ultracentrifugation and CD9-immunobeads isolation kit. We decided to isolate exosomes from HeLa and Caski cell lines supernatant (HPV18 and HPV16 positive, respectively) and from patient serum, urine and liquor HPV positive.

In addition, we aim to understand if viral nucleic acid transfer mechanism, can be extended to other viruses, such as polyomavirus. SV40 polyomavirus was find in different cancer, including medulloblastomas, breast cancer, mesotheliomas (Vilchez RA et al., 2003), but few information are available about its role in human tumors and its route of transmission. Our unpublished data demonstrated the presence of SV40 DNA positive in bone tumors. We plan to use SV40 positive exosomes, isolated by COS7 cell line supernatant (SV40 positive cell line).

Exosomes obtained will be used to test the hypothesis of viral nucleic acid transfer to recipient cells. First, we will analyze their acid nucleic and protein content. Then, we will quantify their number by using Nanosight, that could allow us to perform in vitro experiment by using the same exosomes number. We will test different recipient cell lines to verify our hypothesis. Exosomes exposed cells will be analyzed at different time point of exposure by using Real-time PCR, conventional PCR and digital PCR.

3. MATERIAL AND METHODS

Cell lines and cultures

MCF7 human breast cancer cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy) and 1% penicillin/streptomycin at 37°C in 5% CO_2 humidified atmosphere.

Caski and HeLa cervix cell lines, contain Human Papilloma Virus (HPV), HPV16 and HPV18 respectively. They were cultured in RPMI1640 and DMEM, respectively, supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy) and 1% penicillin/streptomycin at 37°C in 5% CO_2 humidified atmosphere.

COS7 cell line, contains SV40 viral DNA sequences and it was cultiured in DMEM supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy) and 1% penicillin/streptomycin at 37°C in 5% CO_2 humidified atmosphere.

HCT116 and NCM460 (colon cell lines), H2052, SAOS2 and U2OS (mesothelioma and osteosarcoma cell lines), COS7 (SV40 trasformed cell line) and A172 (glioblastoma cell line), were cultured in DMEM and RPMI1640, supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy) and 1% penicillin/streptomycin at 37°C in 5% CO_2 humidified atmosphere.

Normal and Tumoral Human Mammary Fibroblast (NHMF and THMF) were obtained from breast patient tissue. After collagenase/hyaluronidase digestion, cells were centrifuged at 500 *g* for 5 min and the pellet containing fibroblasts was suspended in DMEM medium supplemented with 20% fetal bovine serum (FBS) (Euroclone, Milan, Italy), penicillin–streptomycin and glutamine (Sigma). Hypoxia was obtained by exposing cells to 1% pO_2, 5% pCO_2, 94% pN_2 gas mixtures in an in vivo 300 Hypoxia Cabinet (Ruskinn Technology, Bridgend, UK).

Mammospheres generation from normal tumor human breast tissues

Tumor samples (ductal breast carcinoma) were separated from the surrounding normal tissue, under sterile conditions, and were diagnosed as normal or neoplastic, following standard diagnostic procedures. Normal and tumor tissues were processed to generate MS. Cells were filtered through a 40 μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ) and were suspended in 1.5 cm^2 low attachment wells (Becton Dickinson) with 1 ml of complete Mammary Epithelial Growth Medium (MEGM) supplemented with B27, Epidermal and Fibroblast Growth Factors, Insulin and Hydrocortisone (Lonza, Basel, Switzerland). Primary MS started forming after 4–6 days and

processed for secondary MS. The procedure was approved by the local ethical committee and by the patients written informed consent.

Specimens

This study was approved by the local ethics committe (number 145/2015/U/Sper; number 006/2012/U/Tess). All patients gave written informed consent in accordance with the Declaration of Helsinki.

Exosome isolation

Differential Ultracentrifugation

Exosomes were isolated from normoxic and hypoxic cells-conditioned medium, and from serum-derived patient, as recently reported (King et al., 2012). HeLa, Caski and COS7 cells were cultured in medium serum free, for 48 h. Briefly, the medium or serum patient, were first centrifuged at 500*g* for 10 min to spin down intact cells and next at 20.000*g* for 20 min to pellet cell debris. Finally, the supernatant or serum were ultracentrifugated at 100.000*g* for 120 min to obtain the exosome pellet, which was washed in PBS and centrifuged again at 100.000*g* for 120 min. Exosome pellet was re-suspended in 50 µl of PBS. Exosomes were DNASE 1 digested **(**DNase I RNase-free 1 U/µL, Thermo Fisher, EN0521**)** prior to nucleic acid extraction.

CD9 immunobeads isolation kit

Exosome biologic fluids, including serum, urine and liquor were isolated by CD9 immunobeads isolation kit (Hansa-Biomed) according to the manufacturer's protocol. Briefly, biological fluids samples, were first subjected to different centrifugation to spin down cell debris. 10 µl of immunobeads were added to samples, and were incabate 4°C overnight in rotator. The day after, samples were subjected to several centrifugated of 5000 rpm for 10 min, to collect the CD9 negative fraction. Immunobeads were resuspended and centrifugated twice 5000 rpm for 10 min, with 50 µl of exosomes eluition buffer to collect two CD9 positive fractions.
Exosomes can be used to make in vitro transfers or can be analyzed at molecular level: they are resuspended with RIPA buffer for protein extraction, TRIzol® (Life Technologies, Milano, Italy) for total RNA extraction, or Lysis Buffer for DNA extraction.

Exosome transfer

Exosomes obtained by differential ultracentrifugation or CD9-immunobeads isolation kit, were analyzed by using the NS500 nanoparticle characterization system (Nanosight Malvern Instruments) thanks to collaboration with IRST (Meldola). This instrument allows to misure exosomes distribution, number/ml and mean size plotted as a diagram file. Exosomes quantification allow us to performed in vitro experiments with the same vesicles number. We exposed cells to the same vesicles number and we analyzed acid nucleic transfer at different timepoint.

RNA extraction with Trizol

Cells and exosomes RNA extraction were performed by Trizol protocol (Life Technologies, Milano, Italy). Pellet cell or exosomes were resuspended in 400 µl of Trizol and mixed. 160 µl of chloroform were added, and incubated for 5' at RT. After centrifugation at 12,000xg for 15' at 4°C, the upper phase was transferred to a new fresh tube. 200 µl of isopropanol and 2 µl of glycogen were added to the sample and mixed by inversion several times, and incubated for 10' at 4°C. The samples were then centrifuged at 12,000xg for 15' at 4°C. The supernatant was discarded and the pellet washed in 500 µl of 75% EtOH. The RNA was pelleted with a centrifugation at 12,000xg for 10' at 4°C, air dried and resuspended in 12 µl of DEPC H2O. RNA was stored at -80°C.
The RNA concentration was measured by Thermo Scientific NanoDrop™ 1000 Spectrophotometer.

DNA extraction

Cells, exosomes and formalin-fixed, paraffin-embedded (FFPE) tissues samples DNA extraction were performed by Nucleo Spin Tissue or Nucleo Spin FFPE Tissue (Machery-Nagel) respectively. Pellet cells, exosomes and FFPEs samples were resuspended in lysis buffer and proteinase k and were incubated for 10' to 70°C (pellet cells and exosomes) or overnight to 70°C (FFPEs samples). Samples were transferred on the appropriate columns and centrifugated at 11000g for 1'. The supernatants were discarded and samples were washed and centrifugated at 11000g for 1' again. This step was repeated three times. DNA was eluted with 30-100 µl of eluition buffer. Dna concentration was measured by Thermo Scientific NanoDrop™ 1000 Spectrophotometer and stored at -30°C.

Real-time PCR on DNA and cDNA

An amount of 0.1-1 µg of total RNA was retro-transcribed by using the Master RT plus PCR system kit (LifeTechnologies). The reaction is composed by:

STEP 1:

REAGENT	**VOLUME (µL)**
Oligo dt or Random Primers	1
dNTPs	1
RNA	0.1 – 1 µg
H20	Up to 12 µL
	VOLUME FINAL: 12 µL

STEP 2:

REAGENT	**VOLUME (µL)**
Buffer 5x	4
DTT	2
MMLV	1
Rnase Out	1
	VOLUME FINAL: 8 µL

The program machine is:

STEP 1	**TEMPERATURE**	**DURATION**	**CYCLES**
	65°C	5 min	1
	4°C	for ever	

STEP 2	**TEMPERATURE**	**DURATION**	**CYCLES**
	37°C	50 min	1
	72°c	15 min	1
	4°C	for ever	

cDNA and DNA were amplified by Syber Green (SYBR® Select Master Mix for CFX, Life Technologies) or Taqman (Taqman Universal Master Mix, Life Technologies) approaches, by using Mx3000P Stratagene instrument (Thermo Fisher) following the manufacturer's instruction. The data was analyzed by using the $2^{-\Delta\Delta CT}$ method. The beta-glucuronidase and Beta-Actin, were used as endogenous controls for cDNA or DNA analysis, respectively. Each sample was analyzed in replicate. All primers used in Real-time PCR are listed in table.

REAGENT	VOLUME (µL)
Taqman Universal Master Mix (2x) / SYBR® Select Master Mix for CFX (2x)	5
Probe Taqman/ Primer Fw and Primer Rev	Variable
Sample + H2O	Variable
	VOLUME FINAL: 10 µL

Real-time TAQMAN program machine:

STEP	TEMPERATURE	DURATION	CYCLES
Udg activation	50°C	2 min	1
Amplitaq DNA polymerase activation	95°c	2 min	1
Denature	95°C	15 sec	45
Anneal/Extend	60°C	1 min	

Real-time Syber Green program machine:

Primer TM > 60°C

STEP	TEMPERATURE	DURATION	CYCLES
Udg activation	50°C	2 min	1
Amplitaq DNA polymerase activation	95°c	2 min	1
Denature	95°C	15 sec	45
Anneal/Extend	60°C	1 min	

Primer TM < 60°C

STEP	TEMPERATURE	DURATION	CYCLES
Udg activation	50°C	2 min	hold
Amplitaq DNA polymerase activation	95°c	2 min	hold
Denature	95°C	15 sec	45
Anneal	55-60°C	15 sec	
Extend	72°C	1 min	

Pcr standard and nested

DNA and cDna were amplified by a PCR standard reaction using a T100 Thermal cycler Bio-Rad or GeneAmp® PCR System 9700 (Applied Byosistem) machines. Each amplification reaction was performed by GoTaq®Flexi DNA Polymerase kit (Promega). Pcr products were detected on 1.8% agarose gel by using ChemiDoc™ XRS+ System (Bio-Rad). Nested pcr was performed to increase the sensitivity and/or specificity of PCR. The primers used in the first round of amplification are either both replaced for the second and subsequent cycles of amplification. In alternative, the primers used for the second amplification, are internal to the original primers used to generate that amplicon. All primers are listed in table.

PCR MIX REACTION:

REAGENT	**VOLUME (µL)**
5X Colorless GoTaq® master mix 5	5
25 mM MgCl2 2	2
10 µM dNTPs mix	1
10 µM Primers mix	0.5
5 U/µl GoTaq®Hot Start Polymerase	0.125
Sample	Variable
H20	Variable
	FINAL VOLUME = 25 µL

PCR Amplification Program:

STEP	**TEMPERATURE**	**DURATION**	**CYCLES**
GoTaq®Hot Start Polymerase Activation	95°C	2 min	1
Denaturation	95°c	2 min	35-45
Annealing	60°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	7 min	1
	4°C	Hold	

Western Blot

Total proteins were extracted with RIPA Buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, SDS 0.1%, Na-Deoxycholate 0.5%) added with Protease Inhibitor Cocktail (Roche, Basel,

Switzerland). The pellet was re-suspended in RIPA buffer and incubated on ice for 20 min; next the supernatant was recovered by centrifugation at 13.000*g* for 15 min. Proteins were analyzed by Western blot using the following antibodies: anti-CD81 (clone MA5-13548, ThermoFisher); anti-CD63 (MEM-259, Thermo Fisher), anti-CD9 (clone MA1-80307; Invitrogen), anti-Alix (clone 3A9**,** MCA2493, Biorad).

Chromogenic in Situ Hybridization (CISH)

Chromogenic in Situ Hybridization (CISH) was used to detect HPV DNA in tissues. CISH was performed by using the ZytoFast®Plus CISH Implementation Kit HRP-AEC (ZytoVision) using digoxigenin-labeled probes according to manufacturer's protocol. The presence of HPV DNA in tissues was analyzed by using a probe able to recognize both the HPV16/18 DNA (ZytoFast®HPV type 16/18 Probe Digoxigeninlabeled). A probe specific for the ALU sequences, provided by the kit, was used as a positive control (ZytoFast®DNA (+) Control Probe).

Digital Pcr

Some DNA samples, were analyzed by Digital PCR (QuantStudio® 3D Digital PCR System, Life Technologies), to determine absolute quantification of viral copy number. To detect target sequence were used Taqman assay or primers with specific UPL probe (Roche). Standards or endogenous controls were not used because only target sequences were detected. The amplification reaction is composed by a mixture of sample, master mix and probes. They were coated on chip, through a nanofluidic and automatic system. Chip containing sample, was covered by oil, closed and amplified by using QuantStudio® 3D Digital PCR System. Chip is read and subsequently analyzed by using its software. To account for wells that may have received more than one molecule of the target sequence, a correction factor is applied using the Poisson model. All primers used for dPCR assay are in table.

Statistical analysis

Statistical analysis was performed by SPSS software v10 (SPSS, Chicago, IL). Quantitative variables were assessed by student's t test. P-values were adjusted for multiple comparisons when n>2 samples were compared.

Table 1. Housekeeping primers

GENE	SEQUENCE	POSITION	LENGHT	ASSAY	TARGET
ACTB	CCACACTGTGCCCATCTACG	F 1456	98 bp	Real-time PCR	DNA
	AGGATCTTCATGAGGTAGTCAGTCAG	R 1358			
ACTB	Taqman probe (Life Technologies)			Real-time PCR/Digital PCR	DNA
GUS	Taqman probe (Life Technologies)			Real-time PCR	RNA

Tabella 2. HPV18 primers

GENE	SEQUENCE	POSITION	LENGHT	ASSAY	TARGET
E7 HPV18	GAAAGCTCAGCAGACGACCT	F 818	63 bp	Real-time PCR	DNA
	CACAAAGGACAGGGTGTTCA	R 880			

Tabella 3. HPV31 primers

GENE	SEQUENCE	POSITION	LENGHT	ASSAY	TARGET
E6/E7 HPV31	GTGGACAGGACGTTGCATAG	F 818	185 bp	Real-time PCR	DNA
	GCTGGACTGTCTATGACATC	R 880			

Tabella 4. HPV16 primers

GENE	SEQUENCE	POSITION	LENGHT	ASSAY	TARGET
E6 HPV16	CAACAGTTACTGCGACGTGAG	F 206	349 bp	PCR	DNA
	GCTGGGTTTCTCTACGTGTTC	R 554			
E7 HPV16	CAACTGATCTCTACTGTTATGAGCAA	F 617	73 bp	Real-time PCR/ Digital PCR	DNA
	CCAGCTGGACCATCTATTTCA	R 689			
E1 HPV16	Taqman probe (Life Technologies)			Real-time PCR/Digital PCR	DNA

Tabella 5. SV40 primers

GENE	SEQUENCE	POSITION	LENGHT	ASSAY	TARGET
Large T SV40	TCAGCAGAGCCTGTAGAAC	F 3670	107 bp	PCR	DNA
	AAGCGGGTTGATAGCCTAC	R 3776			
Large T SV40	GCCTCATCATCACTAGATGC	F 4471	468 bp	PCR	DNA
	AGGCTTCTGGGATGCAACTG	R 4939			

4. RESULTS

HPV AND BREAST CANCER

On the basis of meta-analysis studies (Bae JM, 2016) about the presence of HPV in breast cancer patients, we hypothesized that the virus could occur in these tissues. 273 FFPE breast cancer tissues were analyzed by MALDI-TOF mass-spectrometry (Cricca et al., 2015). In particular, the samples were subjected to 16 mucosal HPVs genotypes analysis, that included high and medium risk genotypes. We found 30% of HPV DNA positive samples, including 20% of HPV16 single infection, 7% of other genotypes (18,31,33,35,53,58,68) and 3% of multiple infections with HPV16 (Figure 1).

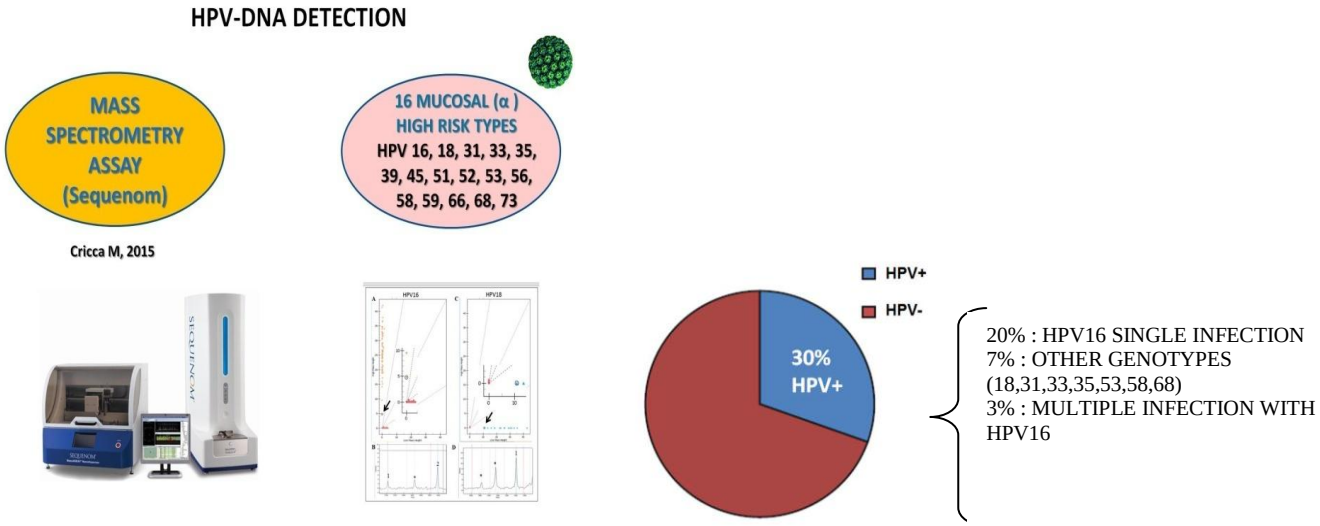


Figure 1. Maldi-TOF mass spectrometry (MS)-based method analysis for 16 mucosal high risk HPV genotypes. Analysis shows 30% of HPV positive samples, including 20% of HPV16 genotype single infection prevalence.

The presence of HPV DNA was also observed and confirmed with in situ hybridization assay (ISH) (Figure 2).

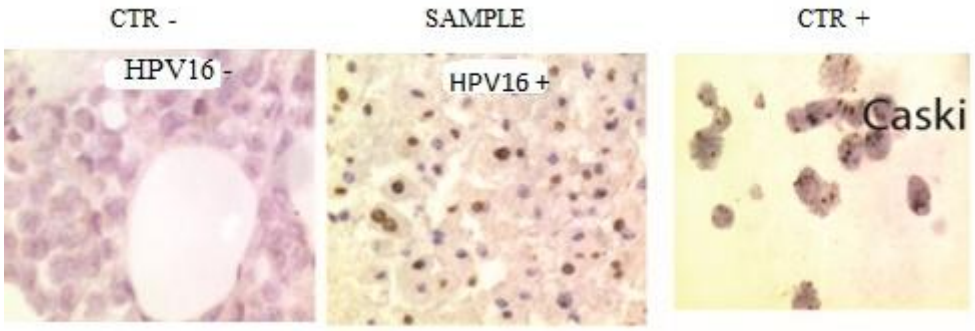
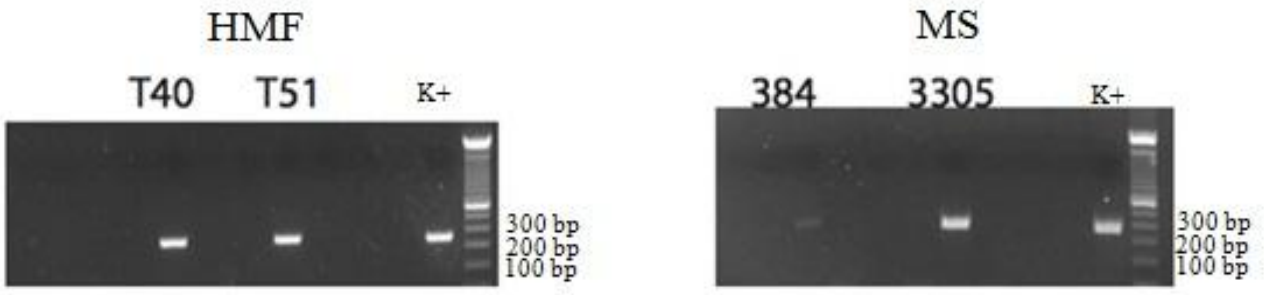


Figure 2: ISH assay shows HPV16 positive tissue.

Two HPV 16 DNA positive as well as 7 HPV DNA negative breast cancer tissues, belonged to a case set of patients whose mammospheres (MS) and human mammary fibroblasts (HMF) had been previously isolated (Papi et al., 2014). We were able to find HPV16 DNA in both MS and HMF from the HPV DNA positive tissues but not from HPV DNA negative ones. One out of the two HMF HPV16 DNA+ was long-term cultured up to passage twelve. The HMF HPV16+ DNA was analyzed at 3, 6 and 12 cell passages for the presence of HPV16 DNA, through the Real-Time PCR (Figure 3A and 3B).

A



B

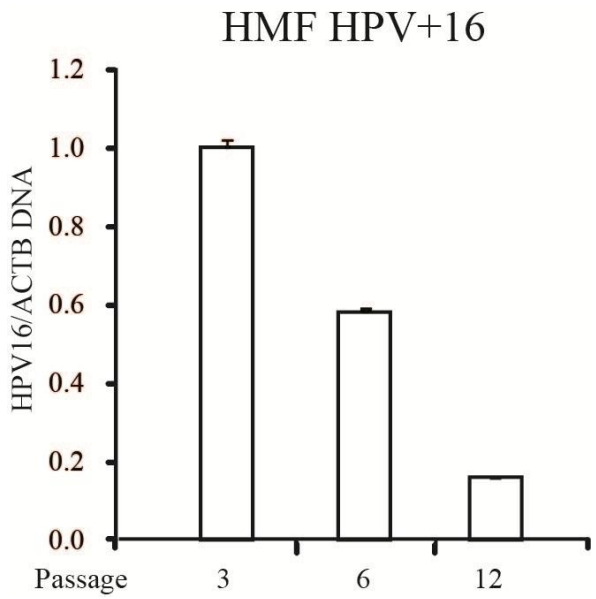


Figure 3. **A:** PCR analysis of HPV16 DNA in 9 MS and HMF breast cancer specimens; 2 out of 9 HMF and MS result HPV DNA positive for 16 genotype. **B:** Real-time PCR analysis of HPV16 DNA in HMF HPV16+ at 3, 6 and 12 passages.

HPV DNA+ serum-derived exosomes from breast cancer patients

We have isolated exosomes from 59 serum specimens from breast cancer patients by differential ultracentrifugation method and we searched for the presence of HPV DNA by a Real-Time PCR assay specific for the E7 gene of HPV 16, 18 and 31. We identified 5 HPV DNA+ samples, including 2 HPV16, 1 HPV16/HPV31, 1 HPV18, and 1 HPV31 (Figure 4).

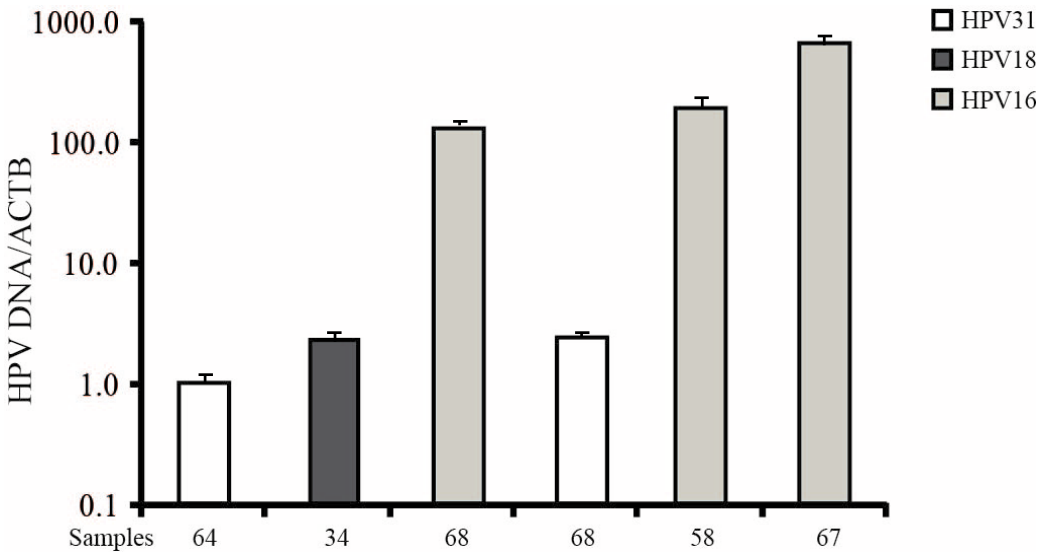


Figura 4. Real-Time PCR analysis of HPV16, HPV18 and HPV31 DNAs in HPV DNA+ serum-derived exosomes.

Subsequently, we analyzed the presence of exosomes markers, i.e CD9, CD63 and CD81 (Figure 5).

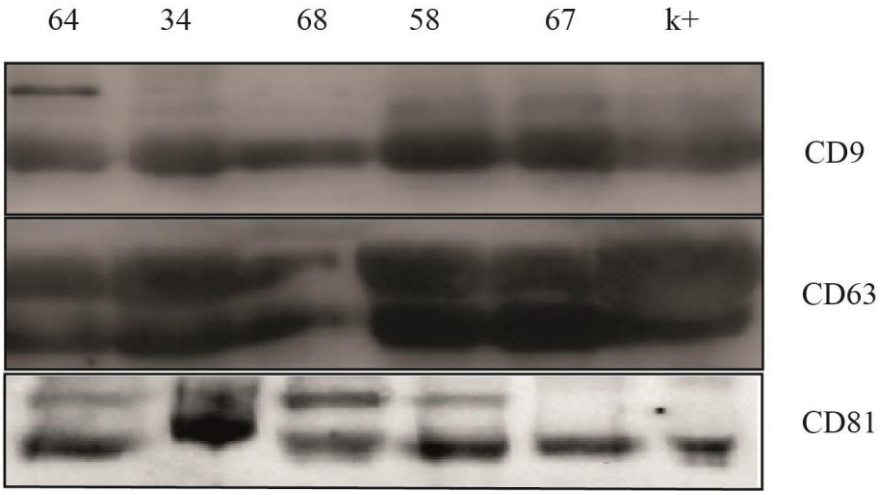


Figure 5. Western Blot analysis of exosomes markers, including CD9, CD63 and CD81.

Viral nucleic acids transfer from HPV DNA + serum-derived exosomes to recipient cells

HPV DNA+ serum-derived exosomes, were used to test in vitro the viral nucleic acid transfer to recipient cells. We exposed MCF7 and HMF, to the HPV DNA+ serum-derived exosomes up to 5 days. We found two HMF HPV16+ samples (Figure 6).

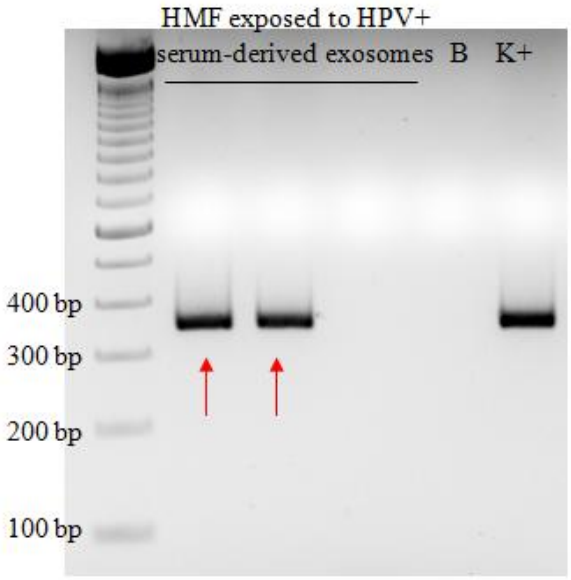


Figure 6. HPV16 DNA analysis of HMF exposed to HPV DNA+ serum-derived exosomes by PCR. Two samples result positive for HPV16 genotype.

Viral nucleic acids transfer from HPV DNA positive cell lines exosomes to recipient cells

We performed our in vitro experiments using the exosomes derived from the cervical HPV DNA positive cell line, Caski (HPV16 positive). We collected 80 ml of serum deprived supernatant and we isolated exosomes by differential ultracentrifugation. The exosomes were purified by filtering at 0.1 and 0.22 μm particle size as well as in absence of any filtration. We evaluated the amount of viral nucleic acids in the exosomes by Real-time PCR (Figure 7).

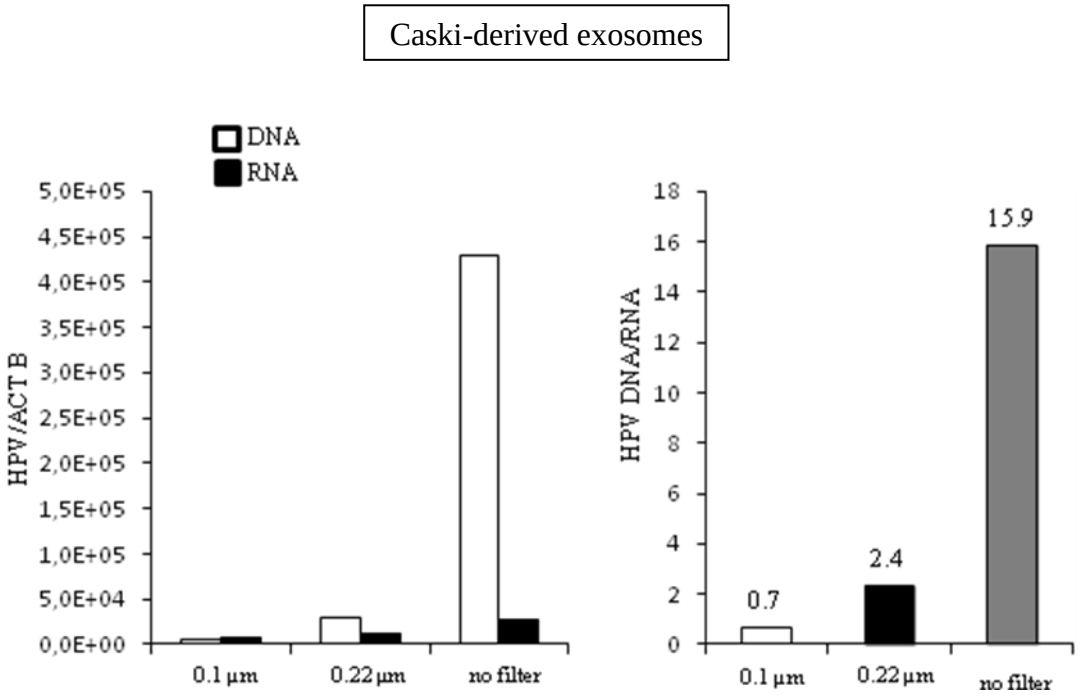


Figure 7. DNA and RNA HPV16 analysis by Real-time PCR of Caski-derived exosomes isolated by differential ultracentrifugation. We used 0.1 μm, 0.2 μm filters and no filters to evaluate HPV acid nucleic content. The analysis shows a significant viral nucleic acid content decrease by using filter.

We analyzed protein content of 0.22 µm or not filter Caski-derived exosomes. We searched for three markers exosomial proteins, including Alix, CD63 and CD9 (Figure 8)

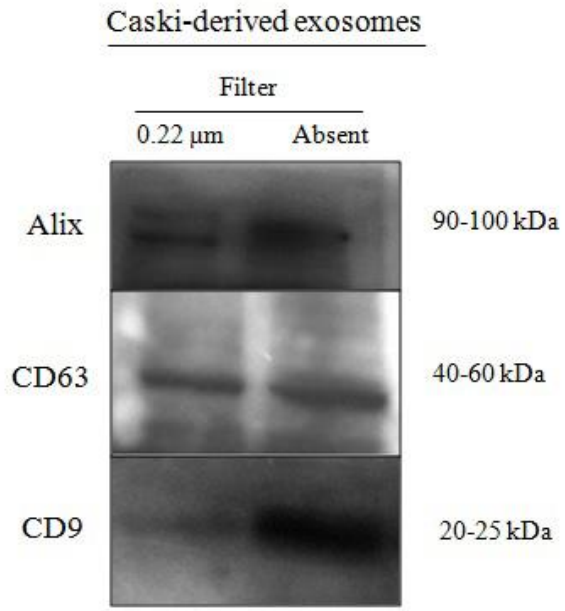


Figure 8. Western Blot analysis of 0.22 µm and no filter Caski-derived exosomes.

Caski-derived exosomes were analyzed by Digital PCR to evaluate the number of HPV16 E1 and E7 gene copies in normoxia and hypoxia condition (1% pO2). We also performed a DNAse 1 digestion prior to nucleic acid extraction to verify the effective presence of HPV DNA inside the exosomes. We showed the presence of HPV DNA also after the DNAse I treatment (Figure 9).

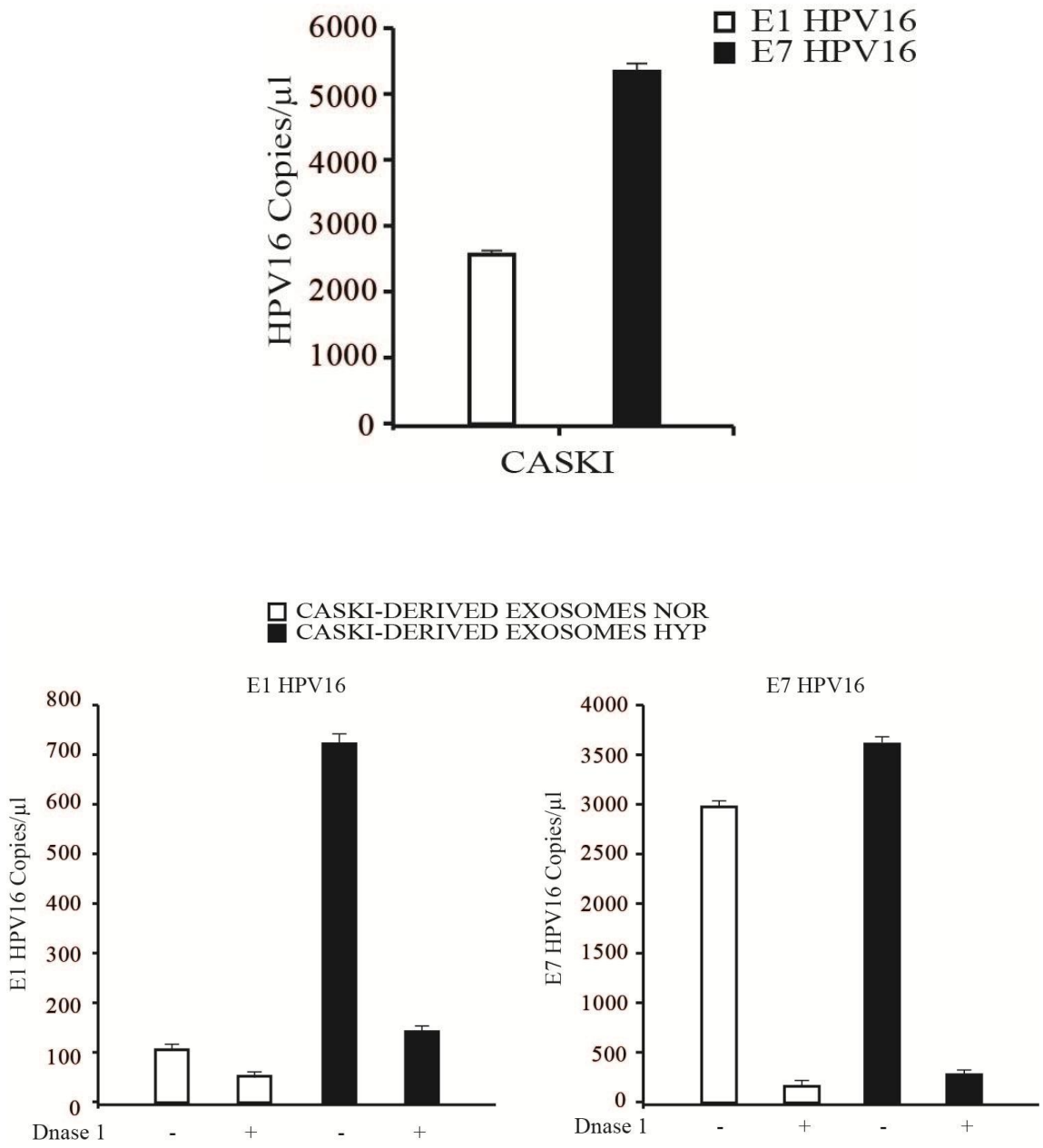
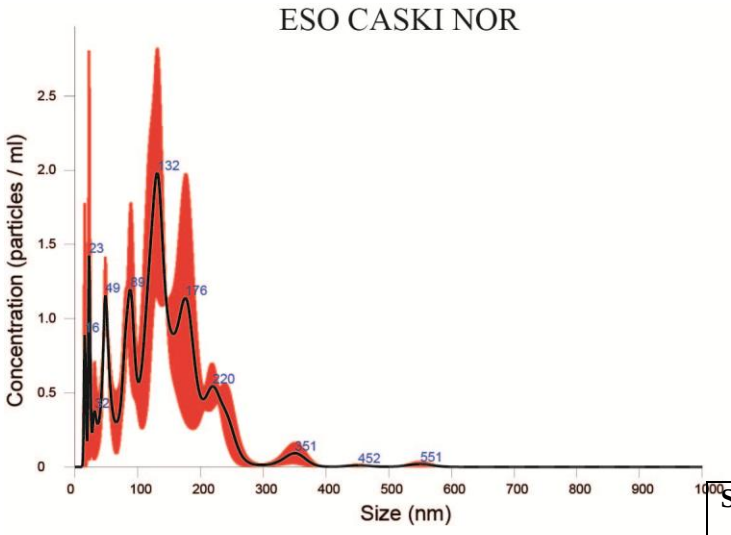
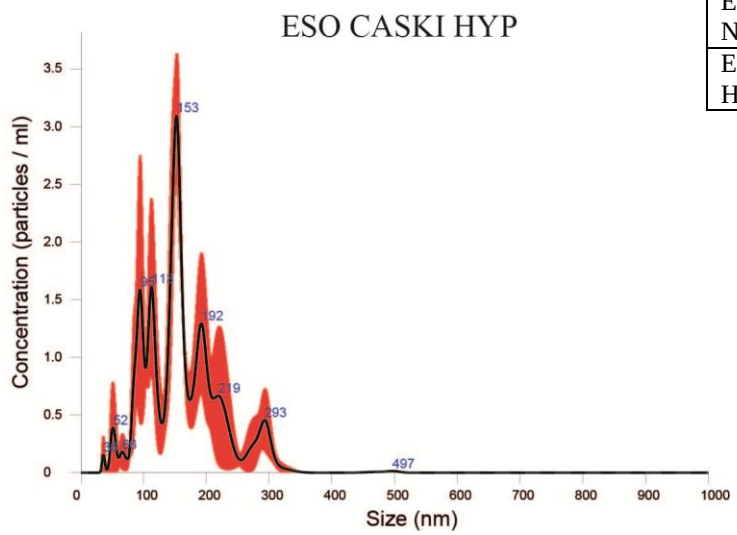


Figure 9: Digital PCR analysis of Caski-derived exosomes. We evaluated both Caski and Caski-derived exosomes DNA viral copies. We isolated Caski-derived exosomes after 48h of normoxic or hypoxic condition. The analysis shows that HPV DNA is still present after DNASE 1 digestion.

We analyzed Caski derived-exosomes by using the NS500 nanoparticle characterization system (NanoSight, Malvern Instruments) in collaboration with Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST, Meldola). This instrument calculates number/ml and mean size of exosomes. It allowed us to use the same number of exosomes in our experiments. Caski derived-exosomes were used to carry out the viral nucleic acid transfer on normal and tumor HMF. We observed an increase of DNA and RNA up to day 7 and a decrease at day 10 of exposure. (Figure 10 A and B)

A





SAMPLE	MEAN (nm)	SD (nm)	PARTICLE/ML
ESO CASKI NOR	140.2	73.3	1.98x10^10
ESO CASKI HYP	161.6	60.8	2.02x10^10

B

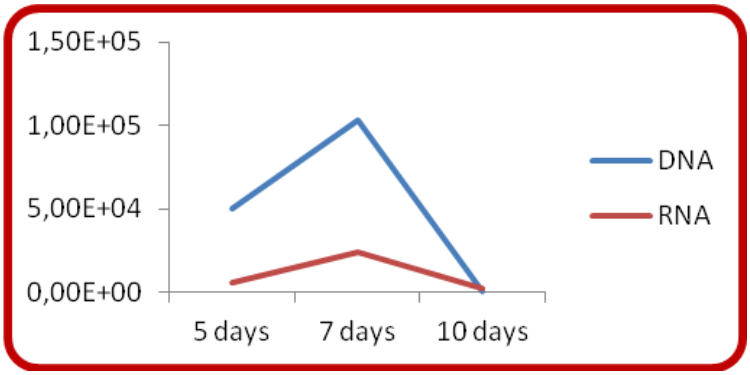


Figura 10. A: Nanosight plot representation of Caski-derived exosomes. **B:** HMF exposed to Caski-derived exosomes was analyzed by PCR assay. This analysis shows that both DNA and RNA are present after 7 day of exposure in exposed to Caski-derived exosomes.

The presence of HPV DNA in HMF exposed to Caski derived-exosomes was evaluated in 4 sets of experiments at day 7 of exposure. Results obtained confirm the presence of HPV16 DNA in normal and tumor fibroblasts (NHMF; THMF) exposed to Caski derived-exosomes, albeit with a little variability of HPV DNA expression (Figure 11).

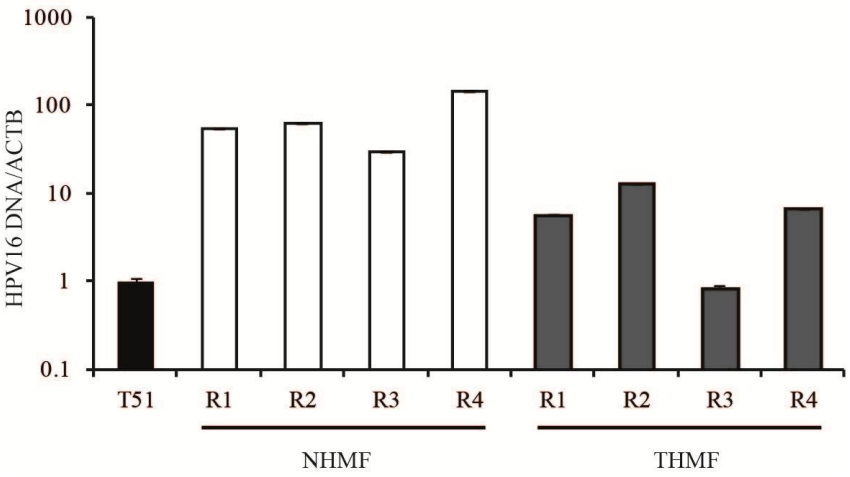


Figure 11: Real-time PCR analysis of HPV16 DNA in NHMF and THMF exposed to Caski derived-exosomes in 4 replicates samples. HPV16 DNA presence is similar among the different replicates. Only THMF R3 and R4 present a little variability of HPV DNA expression.

We analyzed gene expression of some markers about proliferation, inflammation and tumor aggressiveness in NHMF and THMF exposed to Caski derived-exosomes. We observed C-MYC and CYC-D1 expressions increased, that regulate growth and cell proliferation. We also detected an enhancement of Interleukin6 (IL6), especially in exposed THMF. Finally, we observed an increase of CD44 expression in HMF exposed to Caski derived-exosomes, that indicating the activation and the enhanced metabolic activity of fibroblasts (Figure 12).

The increased expression of these genes leads to the assumption that the presence of exosomes in the recipient cell, lead to an aggressive phenotype.

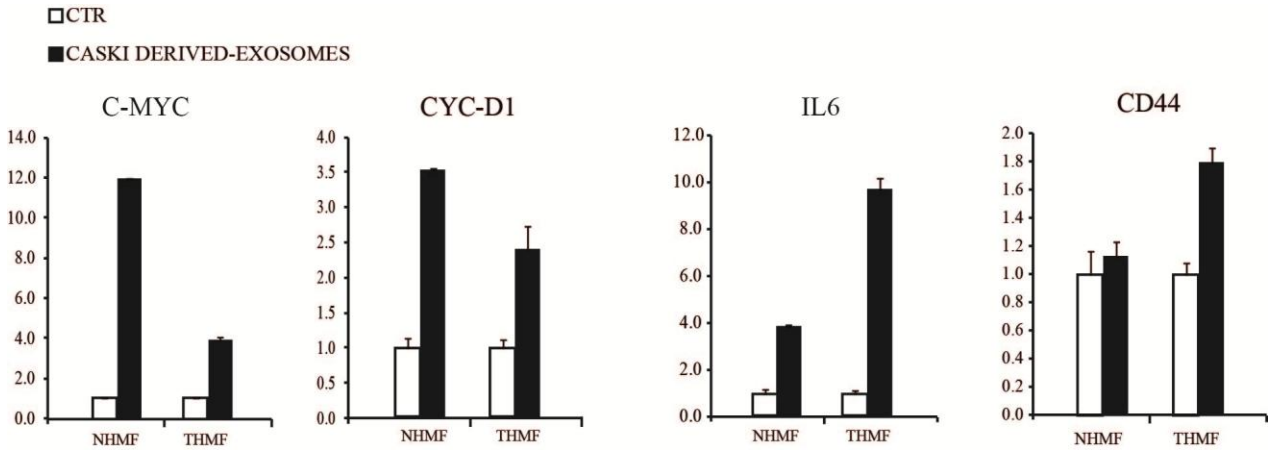


Figure 12: C-MYC, CYC-D1, IL6 and CD44 expressions were analyzed in NHMF and THMF exposed to Caski-derived exosomes by Real-Time PCR. Obtained data show an increased expression of Caski-derived exosomes in NHMF/THMF- exposed to Caski-derived exosomes.

We analyzed 2 out of 4 THMF exposed to Caski derived-exosomes (R3 and R4), characterized by a different content of HPV DNA, to understand whether the increased expression of the genes previously analyzed, depend on the viral nucleic acids presence. We analyzed CD44, IL6; C-MYC and CYC-D1 expressions and we observed an increased of these genes in fibroblast with a higher content of HPV DNA. These analysis indicate that the presence of viral nucleic acids can influence cell phenotype (Figure 13).

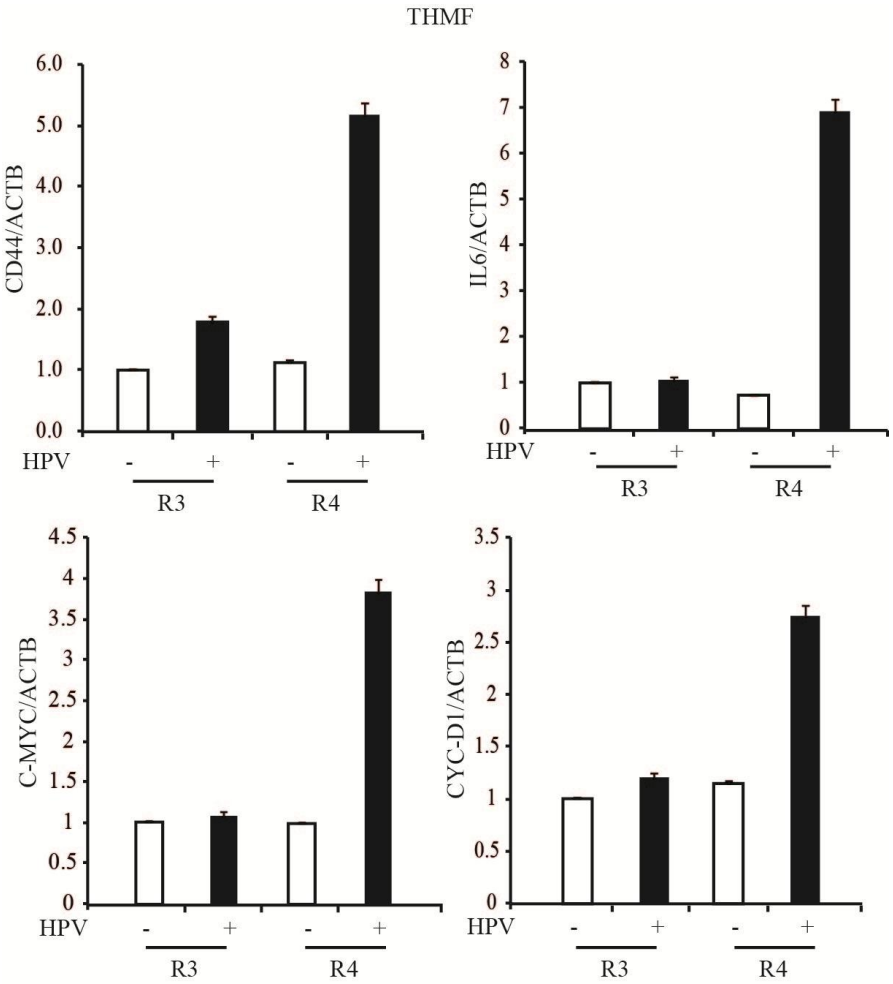
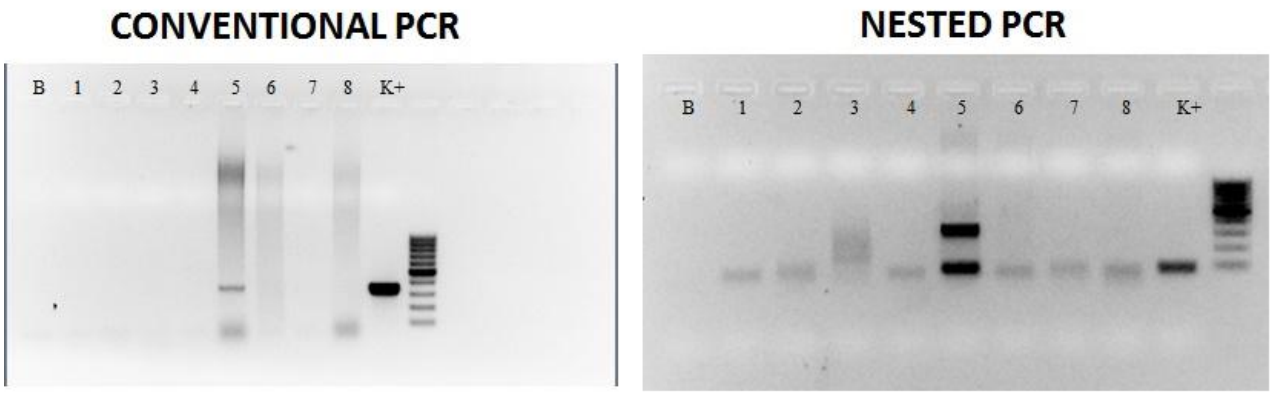


Figure 13: R3 and R4 THMF samples present a different HPV DNA level (Figure 10). We analyzed CD44, IL6, C-MYC, CYC-D1 expression in these samples by Real-time PCR. R4 THMF sample, that presents an higher HPV DNA content, shows an increasead of CD44, IL6, C-MYC and CYC-D1 gene expression, compared to R3 THMF.

We also analyzed DNA from tissue of breast cancer xenograft injected with 10^6 HPV positive exosomes, obtained from a collaboration with a Memorial Sloan Kettering Cancer Center research team. Breast cancer xenograft developed 8 tumors and one of these was found DNA HPV16+ by PCR. Breast cancer xenograft HPV16 positive tissue was analyzed by ISH assay, confirming positivity for this HPV16 genotype (Figure 14 A and B).

A



B

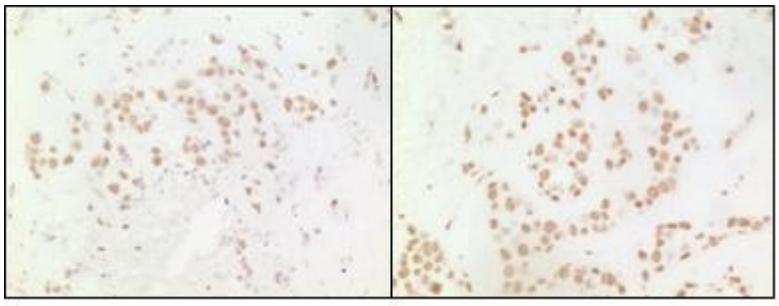
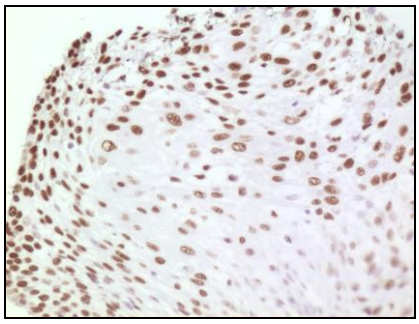


Figure 14: A: Conventional and Nested PCR confirmed HPV16+ DNA in one of breast cancer xenograft tumor tissue (tumor 5); **B:** ISH assay of tumor 5 confirmed HPV16 positive genotype.

CASE REPORT 1: HPV AND ECTOPIC RECTAL SPINOCELLULAR CANCER

We analyzed the presence of viral nucleic acids in the serum-derived exosomes of a patient with an HPV16 positive ectopic rectal spinocellular cancer. We were able to recover the patient's serum, from which we isolated exosomes by CD9-immunobeads isolation kit. We observed the presence of HPV16+ DNA exosomes (Figure 15 A and B).

A



Alphapapillomavirus 9 isolate PAP1263, complete genome
Sequence ID: gb|KU053905.1| Length: 7920 Number of Matches: 1

Range 1: 6586 to 6984 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
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Query  422   TGGTTACCCCAACAAATGCCATTATTGTGGCCCTGTGCT  460
             |||||||||||||||||||||||||||||||||||||||
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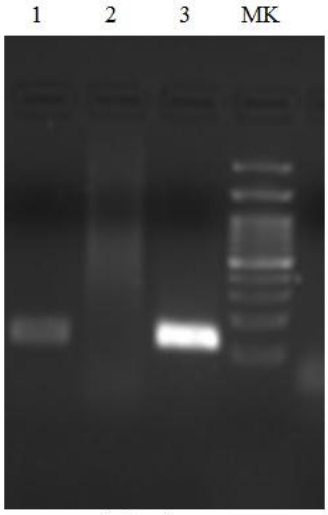
```

B

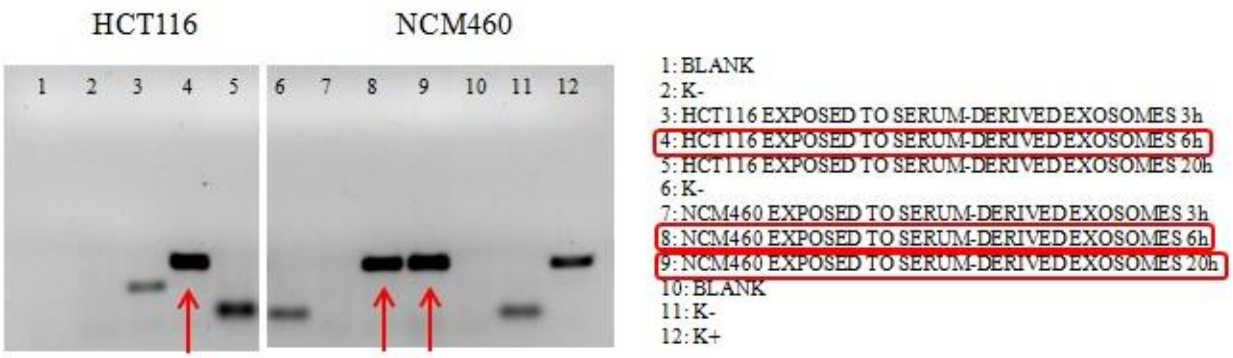


1: Serum-derived exosomes
2: K-
3: K+ (Caski)

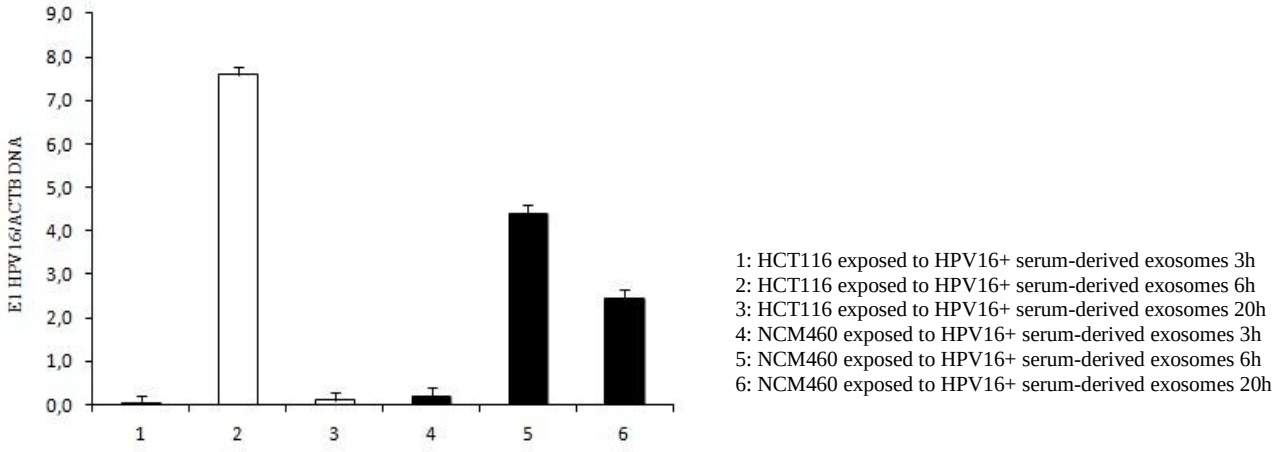
Figura 15. A: ISH assay shows a HPV16 DNA positive mass. We analyzed and sequenced DNA from HPV16+ mass tissue and we confirmed HPV16 positive. **B:** DNA of serum-derived exosomes is analyzed by PCR resulting HPV16 genotype positive.

Subsequently, we used HPV16+ serum-derived exosomes to perform in vitro studies. In particular, we exposed two HPV DNA negative colon cell lines, HCT116 and NCM460, to the patient HPV16+ serum-derived exosomes. We collected the exposed cells at 3, 6 and 20 hours and we found the presence of HPV16 DNA up to 6 hours in both cell lines and up to 20 hours in NCM cells. By Real time PCR we observed a low amount of E1 HPV16 also at 20 hours in HCT and at 3 hours in NCM. We were able to estimate both E1 and E7 HPV16 genes copies in HCT116/NCM460 exposed to HPV16+ serum-derived exosomes by Digital PCR. This analysis shows a low viral content transfer to recipient cell (Figure 16 A, B and C).

A



B



C

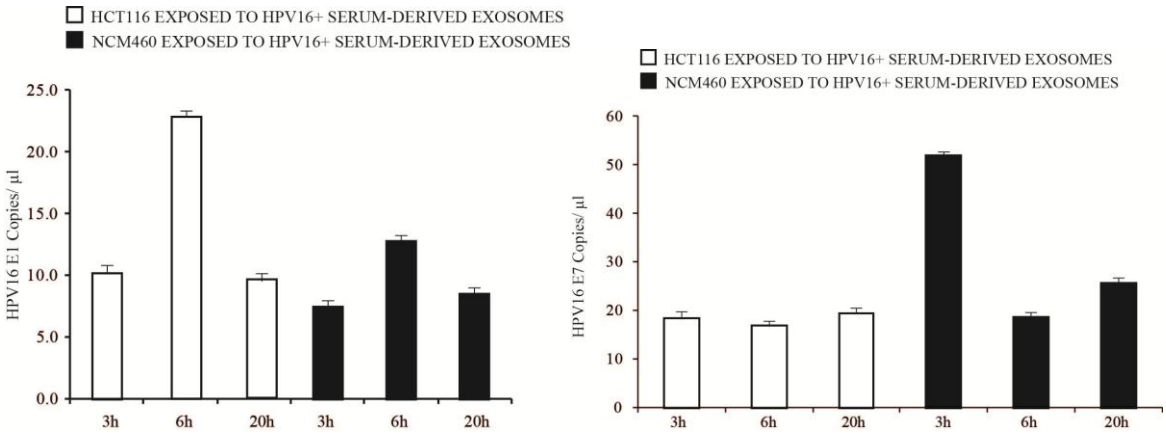
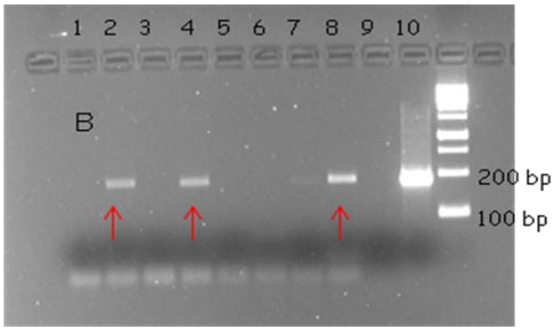


Figure 16. A: We analyzed HPV16 DNA in HCT116 and NCM460 exposed to HPV+ serum-derived exosomes at different time point (3h, 6h and 20h) by PCR. We found HPV16 DNA in both cell lines. **B:** We confirmed PCR data, by using Real-time PCR. We also found E1 HPV16 in HCT116 exposed to 20h and NCM exposed to 3h. **C:** We analyzed E1 and E7 HPV16 DNA viral copies by using Digital PCR. The data obtained show a low HPV DNA copies in both cell lines exposed to HPV+serum-derived exosomes.

CASE REPORT 2: HPV DNA+ ORAL CANCER AND INFLAMMATORY CNS NEUROPATHY OF UNDERMINED ORIGIN

We analyzed the liquor, serum and urine-derived exosomes of a patient with an HPV positive oral cancer who developed an inflammatory CNS neuropathy of undetermined origin. We hypothesized that HPV could be involved in this disorder. We isolated exosomes by CD9-immunobeads isolation kit. Both the CD9+ and the CD9- fractions were analyzed for the presence of HPV DNA and we determined the presence of viral nucleic acids in the CD9+ fractions from serum and liquor specimens. We also determined a low amount of HPV DNA in the CD9– fraction from urine. The positive samples were purified and sequenced and we found HPV16 genotype (Figure 17 A and B).

A



1: Blank
2: Serum-derived exosomes CD9+
3: Serum-derived exosomes CD9-
4: Serum-derived exosomes CD9+
5: Serum-derived exosomes CD9-
6: Urine-derived exosomes CD9+
7: Urine-derived exosomes CD9-
8: Liquor-derived exosomes CD9+
9: Liquor-derived exosomes CD9-
10: K+

B

Human papillomavirus type 16, complete genome
Sequence ID: NC_001526.4 Length: 7906 Number of Matches: 1

Range 1: 5730 to 6077 GenBank Graphics ▼ Next Match ▲ Previous Match

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Sbjct  5777  CAACAACAGTAACAAATAGTTGGTTACCCCAACAAATGCCATTATTGT  5730
```
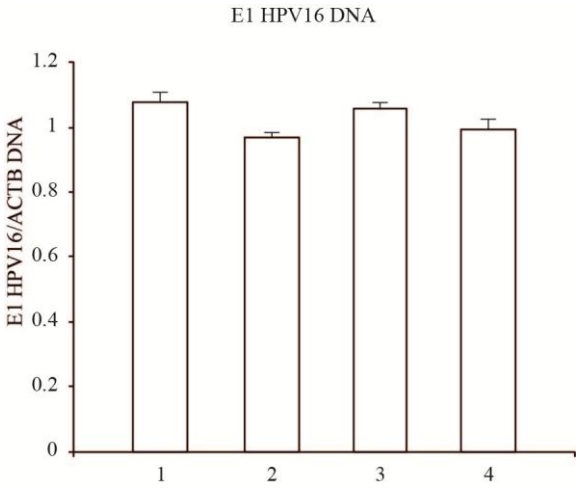
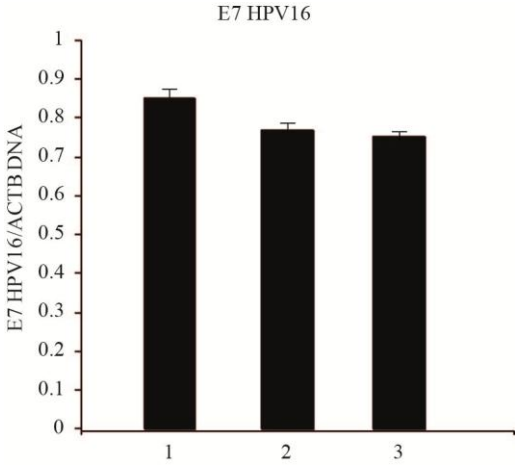

Figure 17. A: HPV degenerate primers (MY09-11) were used to analyzed liquor, serum and urine-derived exosomes by using PCR. **B:** HPV DNA positive sample were sequenced and we found 3 HPV16 positive genotype.

We decided to expose a glial cell line, A172, to Caski derived-exosomes and we analyzed the presence of HPV16 DNA at two different timepoints, 6h and 24h by using PCR, Real-Time PCR and Digital PCR. We observed a low amount of HPV DNA persistence up to 24 hours both by using normoxic and hypoxic exosomes (Figure 18 A, B and C).

A

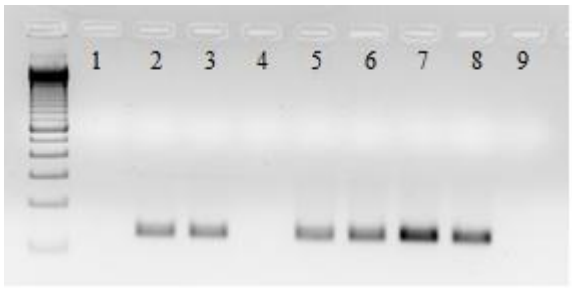


1: A172 EXPOSED TO CASKI-DERIVED EXOSOMES NOR 6h
2: A172 EXPOSED TO CASKI-DERIVED EXOSOMES HYP 6h
3: A172 EXPOSED TO CASKI-DERIVED EXOSOMES NOR 24h
4: A172 EXPOSED TO CASKI-DERIVED EXOSOMES HYP 24h



1: A172 EXPOSED TO CASKI-DERIVED EXOSOMES NOR 6h
2: A172 EXPOSED TO CASKI-DERIVED EXOSOMES HYP 6h
3: A172 EXPOSED TO CASKI-DERIVED EXOSOMES HYP 24h

B



1: K-
2: A172 exposed to Caski-derived exosomes Nor 6h
3: A172 exposed to Caski-derived exosomes Hyp 6h
4: A172 exposed to Caski-derived exosomes Nor 24h
5: A172 exposed to Caski-derived exosomes Hyp 24h
6: Caski-derived exosomes Normoxia
7: Caski-derived exosomes Hypoxia
8: Caski
9: Blank

C

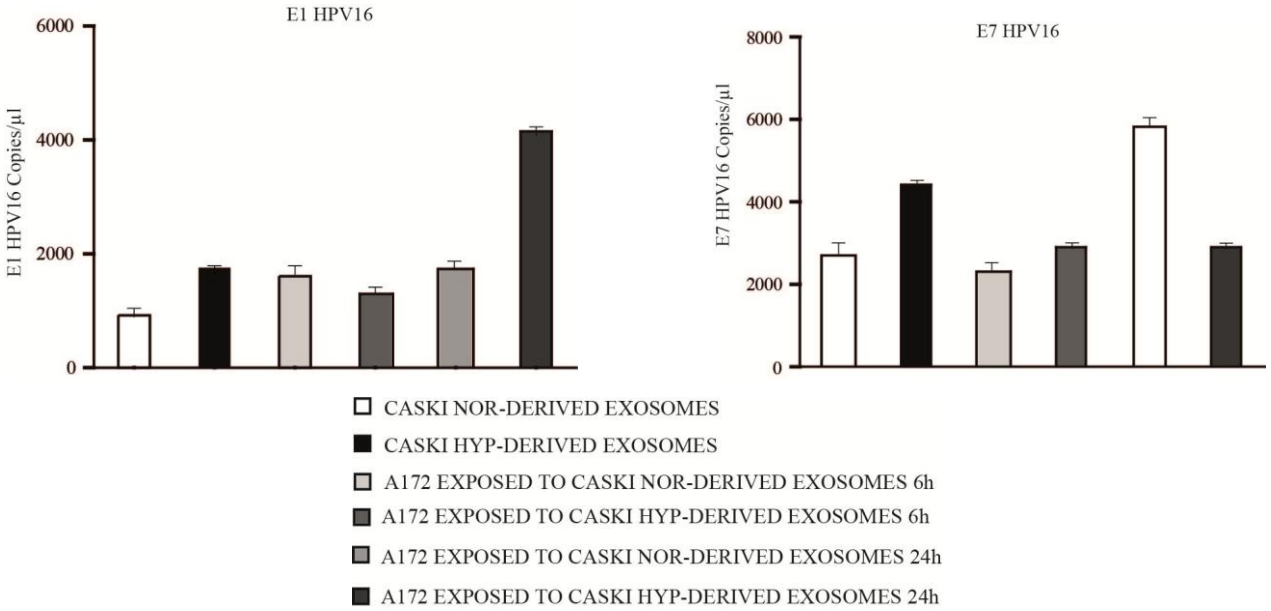


Figure 18. A: We observed both E1 and E7 HPV16 DNA presence in A172 exposed to Caski-derived exosomes, analyzed by Real-time PCR. **B:** Conventional PCR confirmed HPV16 DNA presence in A172 exposed to Caski-derived exosomes up to 20h. **C:** We also evaluated E1 and E7 HPV16 DNA viral copies in A172 exposed to Caski-derived exosomes and we found both E1 and E7 HPV16 copies in recipient cells by using Digital PCR.

POLYOMAVIRUS

We analyzed SV40 DNA in 70 samples of bone tumors and we found 7 positive samples including 4 osteosarcoma (OS), 2 condrosarcoma (CS), and one giant cell tumor (Figure 19).

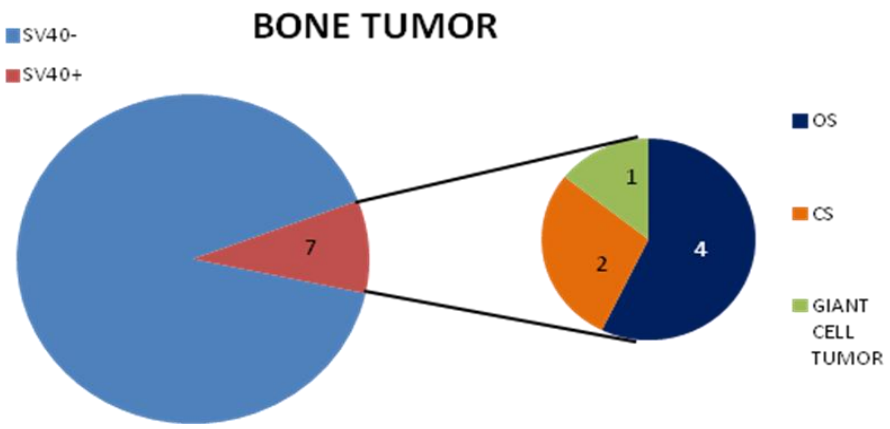


Figure 19: SV40 positive samples in bone tumors.

We isolated COS7-derived exosomes (SV40 positive cell lines), by differential ultracentrifugation. We exposed three osteosarcoma cell lines, U2OS, H2056 and SAOS2 to SV40 DNA positive exosomes. We observed the presence of SV40 DNA in SAOS and H2056 cells up to 7 days and in U2OS in normoxic and hypoxic condition (Figure 20).

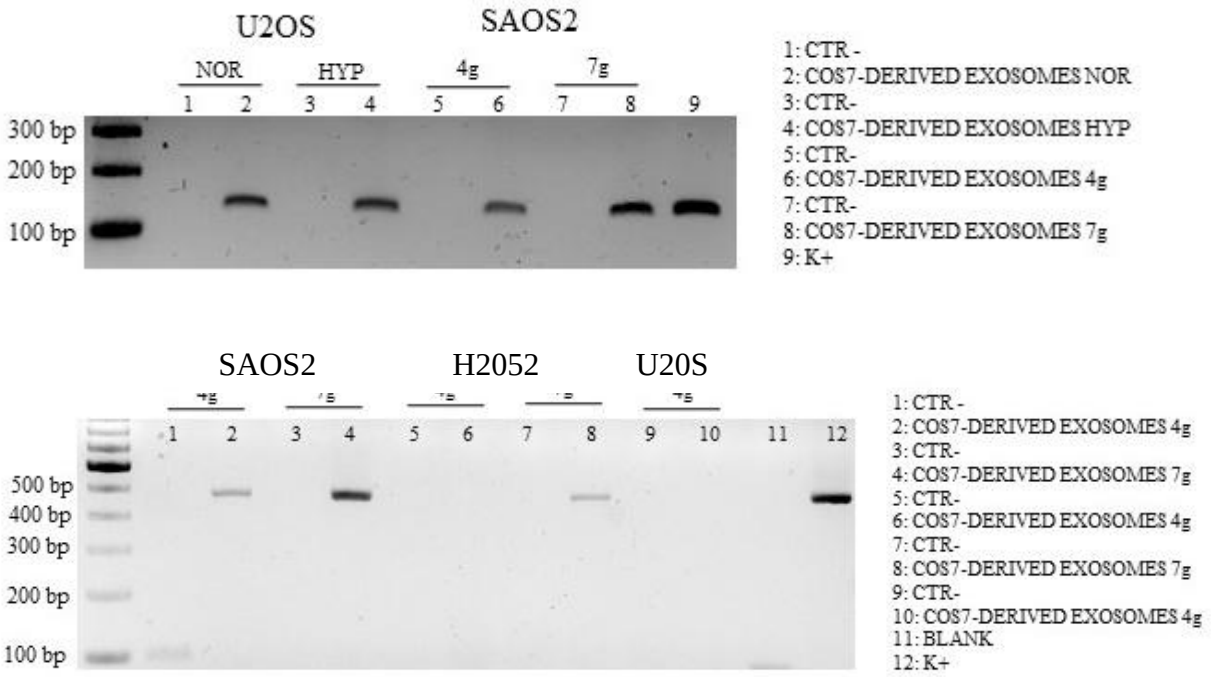


Figure 20. SV40 DNA was detected in SAOS2, H2056 and U2OS exposed to COS7-derived exosomes at different time point and different oxygene percentage condition (normoxic and hypoxic) by PCR assay. We used two different primers set that amplify Large T SV40 regions.

We also observed an overexpression of IFI16, a gene of the interferon system, in COS7-derived exosomes exposed cells. This gene is involved in the natural immune response to the viral nucleic acids entry (Figure 21).

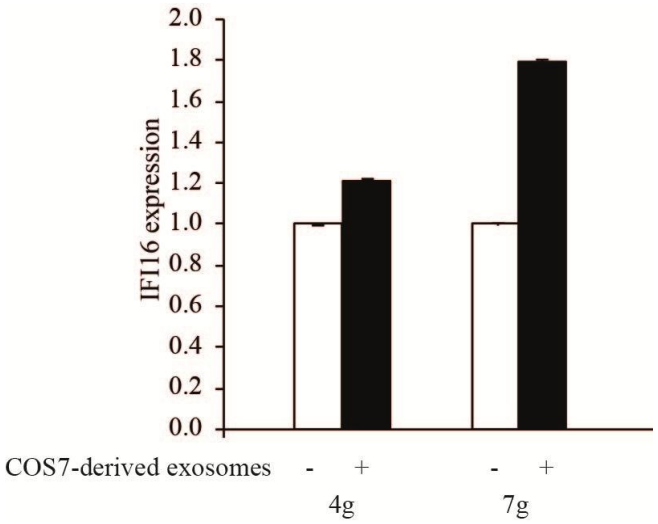


Figure 21. IFI16 expression was found in SAOS2 exposed to COS7-derived exosomes at 4 and 7 day by Real-Time PCR.

DISCUSSION

Breast cancer is a very frequent pathology, mainly in the female population, representing 23% of diagnosed pathologies and 14% of mortality (Jemal A et al., 2011 and Italian Tumor Registry). It is divided according to its molecular and histological characteristics (Lopez LO et al., 2008; Sorlie et al., 2004), which give a greater or lesser aggressiveness to the tumor. Although breast cancer is well characterized, there are some aspects that are still being studied today. In 1992, the first work that described the presence of Human Papillomavirus (HPV) in breast tissue and lymph nodes appeared in the literature, with a prevalence of 29.4% (Di Leonardo A, 1992). Subsequently, meta-analysis studies have illustrated the relationship between HPV and breast cancer (Simoes PW et al., 2012; Bae JM, 2016). In line with the literature, we have searched HPV, in a retrospective case set of 273 breast cancer tissue samples. By a MALDI TOF mass spectrometry assay we found 30% of positive HPV DNA positive samples. The HPV DNA positive tissues, were subjected to further investigation and we were able to observe the presence of HPV by ISH assay. We also isolated HPV16 positive mammospheres (MS) and mammary fibroblasts (HMF) from two HPV DNA positive tissues. We were able to in vitro expand HMF for several passages, demonstrating the persistence of HPV 16 DNA. These data bring to light that the stroma is a district in which not only we would never expect to find out HPV DNA but where HPV is able to "survive" for long time.

HPV is a double-stranded circular DNA virus of about 8000 bp, which represents one of the most frequent sexually transmitted infection (Doorbar J et al., 2012). HPV requires a multilayered mucosal and epithelial cells to replicate, i.e. genital, oral and cutaneous districts, and its complete replicative cycle is strictly dependent by the state of cell differentiation. Owing to the lack of viremia, HPV DNA presence in the mammary gland is still a conundrum. In this regard recent literature reports the presence of circulating HPV DNA in women with cervical lesions (Lawson JC et al., 2016).

Exosomes are small extracellular vesicles of 40-140 nm, released from different types of tissues, cells and biological fluids (Raposo G et al., 2014) and are formed during the composition of multivesicular bodies. Exosomes contain nucleic acids, proteins, non-coding RNAs (Yanez-MO M et al., 2015), and in recent years it has been demonstrated the presence of a viral nucleic acids content (Pegtel DM et al., 2010; Mack M et al., 2000; Esther Nolte-'t Hoen E et al., 2016). We hypothesized that the presence of HPV DNA in the breast tissue could be in relation to the microvesicles and exosomes contents.

On the basis of these observations, we isolated by differential ultracentrifugation exosomes from 59 serum samples of patients suffering from breast cancer, obtaining 5 HPV DNA positive samples, including, HPV16/31, HPV18, HPV31 and two HPV16. These HPV DNA positive exosomes were used to test in vitro the efficacy of HPV DNA transfer in MCF7 and the HFM recipient cells. We observed that the viral DNA was transferred in HFM but not in MCF7 cells. Based on this observation we isolated exosomes by differential ultracentrifugation from the supernatant of HPV DNA positive cell lines (Caski), we evaluated HPV DNA viral copies by using Digital PCR and then we performed in vitro studies to verify the capability of these exosomes to transfer HPV DNA to HFM. We also analyzed exosomes number by using Nanosight and we exposed cells to the same number of exosomes. We analyzed the exposed cells at different time points, noting an increase of HPV DNA up to 7 days of exposure, then HPV DNA tends to decrease. This could suggest that viral nucleic acids cargoed by exosomes, are able to enter into the cell, but obviously need of other conditions to persist in the receiving cell. The delivery of HPV DNA via apoptotic bodies to recipient cells was previously reported by Gaiffe et al. (2012).

Several studies report that exosomes, are responsible for increased cell invasion and proliferation, influencing the phenotype and cellular functions (Yanez-Mo M et al., 2015). Based on these observations, we analyzed some markers responsible for these cellular changes in HFM exposed to HPV positive exosomes. We observed an increase of C-MYC, CYC-D1, IL6 and CD44, especially in samples with a higher HPV DNA content. These data seem to indicate that the entry of HPV+ exosomes in the fibroblast, create a cell failure, bringing the system to become more aggressive and more tumorigenic.

We also found 1/8 HPV DNA positive tumor tissue of breast cancer xenograft injected with HPV positive exosomes, confirmed by ISH assay. These data confirm the role of exosomes as carriers of viral nucleic acids, and the HPV oncogenic activity in extra-genital tissue.

The presence of HPV has been found not only in mammary tumors, but also in other districts, such as lung cancer (Colombara DV et al., 2015), head and neck cancer (Zur Hausen H et al., 2009), colorectal cancer (Yavuzer D et al 2009) and glioblastoma (Vidone M et al., 2014), where the tumor microenvironment, could act as a trigger for a viral infection in an already compromised system.

In this regard, we were able to isolate HPV16 positive serum-derived exosomes by a CD9 immunobeads isolation kit from a patient with an ectopic rectal spinocellular cancer, whose mass was positive for HPV16. We then tried to reproduce what happened in vivo, by exposing the HCT116 and NCM460, two colon cell lines, to HPV16+ serum-derived exosomes. The transfer, showed us, that HPV16 positive serum-derived exosomes release their viral content in these cells.

It has been described that the frequent change of partners and certain sexual habits, may be responsible for the presence of HPV DNA positive lesions of the oral cavity (Rautava J et al., 2012). At this regard we isolate exosomes from serum, urine and liquor specimens (by CD9 immunobeads isolation kit) from a patient who suffered from an HPV DNA positive oral cancer who has presented a CNS neuropathy of undetermined origin with imaging features of inflammatory disease after the removal of the oral cancer. We analyzed the exosomes for HPV DNA and we were able to find HPV16 DNA in the serum and in the liquor. We tried to transfer HPV16 positive Caski-derived exosomes to glia cells (A172) to verify the capability to transfer viral DNA and we noticed a low efficiency of HPV DNA delivery. The obtained data allow us to speculate that the nervous cells are less susceptible to the viral entry.

In last period of my theses I focused my research on SV40 polyomavirus. This polyomavirus is widely studied in the literature and have been associated with different kinds of pathologies (Diamandopoulos GT et al., 1972; Lednický JA et al.,1995; Huang H et al., 1999; Carbone et al., 1996). In particular, SV40 is a virus that was isolated in humans for the first time in the '60s, after the administration of a contaminated polio vaccine. SV40 has been associated with a variety of cancers in animal and also in humans (Vilchez RA et al., 2003). The literature reports the presence of SV40 in medulloblastomas, breast cancer, mesotheliomas but few information are available about its role in human tumors and its route of transmission. Our unpublished data demonstrated the presence of 7 out of 70 SV40 DNA positive bone tumors, including 2 condrosarcoma (CS), 4 osteosarcoma and one giant cell tumor. We isolated by differential ultracentrifugation exosomes from an SV40 positive cell line (COS7) and we performed in vitro experiments to verify their capability to transfer SV40 DNA to recipient cells (U2OS, SAOS2 and H2052). We determined the delivery of SV40 DNA to recipient cells but it was at a very low level of viral copies, indeed we did not obtained the viral transfer in all the replicates.

The entry of viral nucleic acids into recipient cell, involves a series of complex mechanisms, that leading to activation of the interferon system. (Dell'Oste et al., 2015; Lo Cigno et al., 2015). In line with literature, we analyzed and observed an increase of IFI16, in osteosarcoma cells exposed to SV40 positive exosomes. IFI16, would seem to play an important role in the antiviral response, because it is able to recognize viral DNA into the cell, playing a restriction role for viral replication. (Gariano GR et al., 2012)

In light of the obtained data and in line with what has been described in the literature, exosomes may represent a new transport system for viral content, through which viruses can release their nucleic acids in different districts and can evade the immunity system. Furthermore, the release of

the exosomal content, would seem to lead a phenotypic cellular change with more aggressive and tumorigenic characteristics, due to increased inflammation, invasion and cell proliferation.

Exosomes contents, appears to be made up of proteins, viral nucleic acids fragments but also defective viral molecules. Therefore remains to be seen whether the transfer of viral DNA in the recipient cells, may represent a complete replicative unit, triggering a viral infection mechanism.

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