Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
"SCIENZE BIOMEDICHE E BIOTECNOLOGICHE"

CICLO XXX

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Study of association between spontaneous early abortions and infections of polyoma and papilloma viruses

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0. ABBREVIATIONS

ABTS	2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid
BAP1	BRCA1 associated protein 1
BKPyV	BK polyomavirus
CIN	cervical intraepithelial neoplasia
CLL	chronic lymphocytic leukemia
CMV	Cytomegalovirus
CNS	central nervous system
CTB	cytotrophoblast
ddPCR	droplet digital polymerase chain reaction
DEN	Dengue virus
DNA	deoxyribonucleic acid
E1 – E7	gene 1,2,4,5,6,7 of HPV-genome early region
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
enEVTs	endovascular trophoblasts
EVTs	extravillous trophoblasts
GBM	glioblastoma multiforme
hCG	human chorionic gonadotropin
HGF	hepatocyte growth factor
HHV1 (HSV1 - HSV2)	human herpes virus 1 and 2
HIV	human immunodeficiency virus
hPL	human placental lactogen
HPV	human Papillomavirus
HPyV6	human Polyomavirus 6
HPyV7	human Polyomavirus 7
HPyV9	human Polyomavirus 9
hPyVs (PyVs)	human Polyomaviruses
IARC	international agency for research in cancer
iEVTs	interstitial trophoblasts
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G

IgM	immunoglobulin M
IRS-1	insulin receptor substrate 1
JPyV	JC Polyomavirus
KIPyV	Karolinska Institutet Polyomavirus
L1 – L2	gene 1 and 2 of HPV-genome large region
M	miscarriage samples
MCC	Merkel cell carcinoma
MCPyV	MC Polyomavirus
MPM	mesothelioma
MWPyV (HPyV10)	Malawi Polyomavirus
NCCR	non-coding control region
NHL	non-Hodgkin lymphoma
NJPyV-2013 (HPyV13)	New Jersey Polyomavirus 2013
OD	optical density
ORI	origin of replication
OS	osteosarcoma
PAP	Papanicolau test
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PML	progressive multifocal leukoencephalopathy
pRB	retinoblastoma protein
qPCR (real-time PCR)	quantitative polymerase chain reaction
RR	regulatory region of Polyomaviruses
SCC	squamous cell carcinoma
SD	standard deviation
STB	syncytiotrophoblast
STLPyV	Saint Louis Polyomavirus
SV40	Simian virus 40
T-Ag (LT)	large tumor antigen
t-Ag	small tumor antigen

TNF-a	tumor necrosis factor alpha
TSPyV	Trichodysplasia spinulosa-associated Polyomavirus
UM	uveal melanoma
UV	ultraviolet
V.I.	voluntary Interruption samples
VEGF	vascular endothelial growth factor
VP1	major capsid viral protein 1
VP2 – VP3	viral protein 2 and 3
WHO	world health organization
WUPyV	Washington University Polyomavirus
y.o.	years old

1. ABSTRACT

Miscarriages are abortions that occur by natural causes within the twentieth week of pregnancy. Statistically, 80% of these happen in the first trimester of gestation with rates rises from 10% for women at 35 years old and up to 50% for women over the age of 40.
However, other risk factors could interfere with pregnancy, such as autoimmune diseases or vertical pathogen transmissions. Indeed, many infectious agents are associated with spontaneous abortion. There are several studies demonstrating that viral infections are responsible for about 15% of early abortions and 66% of late abortions.
Human Polyomaviruses (hPyVs) are ubiquitous throughout the world's population with infection generally occurring in childhood. The virus then remains latent lifelong in the host. Temporary immunosuppression or -depression may allow various PyV-associated diseases to develop. Traces of PyVs have been identified in different biological specimens like sera, urine and male semen. These data suggest different human-to-human transmission routes, but modes have not yet been completely understood. In particular, vertical transmission has been documented for some Polyomaviruses. This has been shown by viral genomic sequence detection in placenta and fetuses samples.
Up until 2000, both Polyomaviridae family and Papillomaviridae were classified within the Papovaviridae family. Human Papillomavirus (HPV) belongs to the Papillomaviridae family, and literature highlights its ability to replicate in trophoblastic cell lines. This datum, suggests that a vertical transmission may be possible.
This study aimed to investigate the existence of an association between silent intracellular PyVs and HPV infections, and the spontaneous early pregnancy losses. To this purpose, chorionic villi samples (N=98) were collected from miscarriages (M) occurring in the first trimester of pregnancy. These specimens were analyzed in PCR, real-time PCR and Droplet Digital PCR for JCPyV, BKPyV, MCPyV and HPV DNA sequences. Moreover, peripheral blood mononuclear cells (PBMC) from pregnant women were also collected and analyzed (N=92) to evaluate possible vertical transmission. Chorionic villi (N=103) and PBMC (N=102) samples from women undergoing voluntary interruption of pregnancy (V.I.) were also analyzed as the control group.
Overall, results show that there are no statistically significant differences between spontaneous and voluntary abortions. Thus, no association between these kind of infections and early pregnancy losses were found. Nevertheless, JCPyV, BKPyV, MCPyV and HPV were all shown to be able to infect trophoblast. In PBMC, only JCPyV and MCPyV DNA sequences have been identified. All PBMC samples tested BKPyV- and HPV-negative.
JCPyV infection in chorionic villi reached a prevalence of 52% in miscarriages and 59.2% in voluntary abortions. This is the first study that reports on JCPyV-DNA sequences in this kind

of tissue. BKPyV is also able to infect chorionic villi with a prevalence of about 11% both in case and in control cohorts. In contrast, no BKPyV-positivity was found in PBMC samples. Analysis has highlighted MCPyV infections in about 4% (M and V.I.) of trophoblast samples, while PBMC prevalence in M and V.I. samples was 9.8% and 13.7% respectively. Interestingly, in three cases MCPyV was found to have infected both pregnant women's PBMC and the embryo trophoblast. This suggests a possible vertical transmission route. HPV was found to be absent in PBMC from both cohorts but was found in 4% and 3% of M and V.I. chorionic villi, respectively.

Finally, multiple infections were found in about 20% of Polyomaviruses infected trophoblast samples and 100% of HPV-positive tissues were co-infected with JCPyV or MCPyV. These results suggest strong cooperation between different Polyomaviruses as well as between Papilloma- and Polyomaviruses.

2. INTRODUCTION

2.1 Embryo nesting and trophoblast histology

Trophoblast is the external cellular mass of blastocyst, structure which is developed in the first part of the embryogenesis process, after blastocoel formation. It leads to the creation of the placenta and other extra embryonic tissues, but does not participate in forming the embryo itself. The placenta is a transient organ that supports the growth and development of the fetus.

Fertilization of the ovum occurs in the fallopian tubes within 24-48 hours after ovulation. The zygote advances towards the uterus and matures until the morula stage. Then, about 2-3 days after fertilization, the morula enters the uterine cavity (Kunath et al., 2004). Liquid penetrates through the pellucid area and reaches the intercellular spaces of the internal cell mass. This starts to expand, forming an internal cavity called the blastocoel. From this point on, the embryo is called a blastocyst. After that, the outer cells of the blastocyst, which are polarized, give rise to the trophectoderm, while the internal cells, which are not polarized, enable the internal cell mass to be created (Fig. 1).

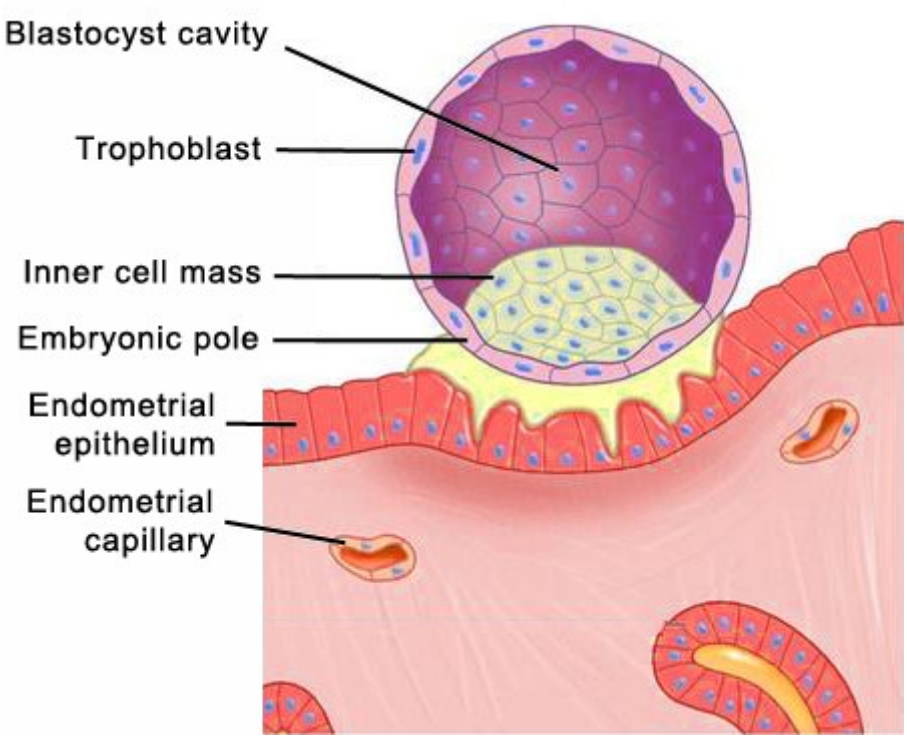

Fig. 1 Blastocyst beginning the infiltration activity on the surface of the Endometrial epithelium. Structure of the blastocyst consists in a monolayer of trophoblast surrounding the blastocyst cavity and the Inner Cell Mass.

About six days after fertilization, embryonic cells communicate with maternal cells to allow blastocyst implantation. Hormones play a very important role in this process (Fazleabas et al., 1999; Norwitz et al., 2001; Paria et al., 2002).

Recent studies have shown that blastocyst nesting in the uterus has many analogies with leukocyte extravasation. Indeed, the L-selectin system plays an important role in rolling and tethering leukocytes and also appears to be involved in trophoblast implantation into the endometrium (Genbacev et al., 2003). At a molecular level, the next phase of trophoblast infiltration is an integrin-dependent process (Damsky et al., 1992; Zhou et al., 1997). At this stage, trophoblast starts to transmigrate across the uterine epithelium, the embryo is completely embedded and the endometrial blood vessels are disrupted (Fig. 1).

The maternal-fetal interface is reduced to a single cell layer by the third trimester (Wang et al., 2010). Defects in the formation of the maternal-fetal interface are associated with various complications that affect pregnancies.

Cytotrophoblast (CTB) is composed of highly proliferating and undifferentiated cells derived from trophectoderm. CTB leads to the differentiation of the trophoblast via two mechanisms. The first involves the fusion of mononuclear CTB cells to form syncytiotrophoblast (STB), consisting of multinucleated cells. STB cover the branched digital structures called chorionic villi (Fig. 2).

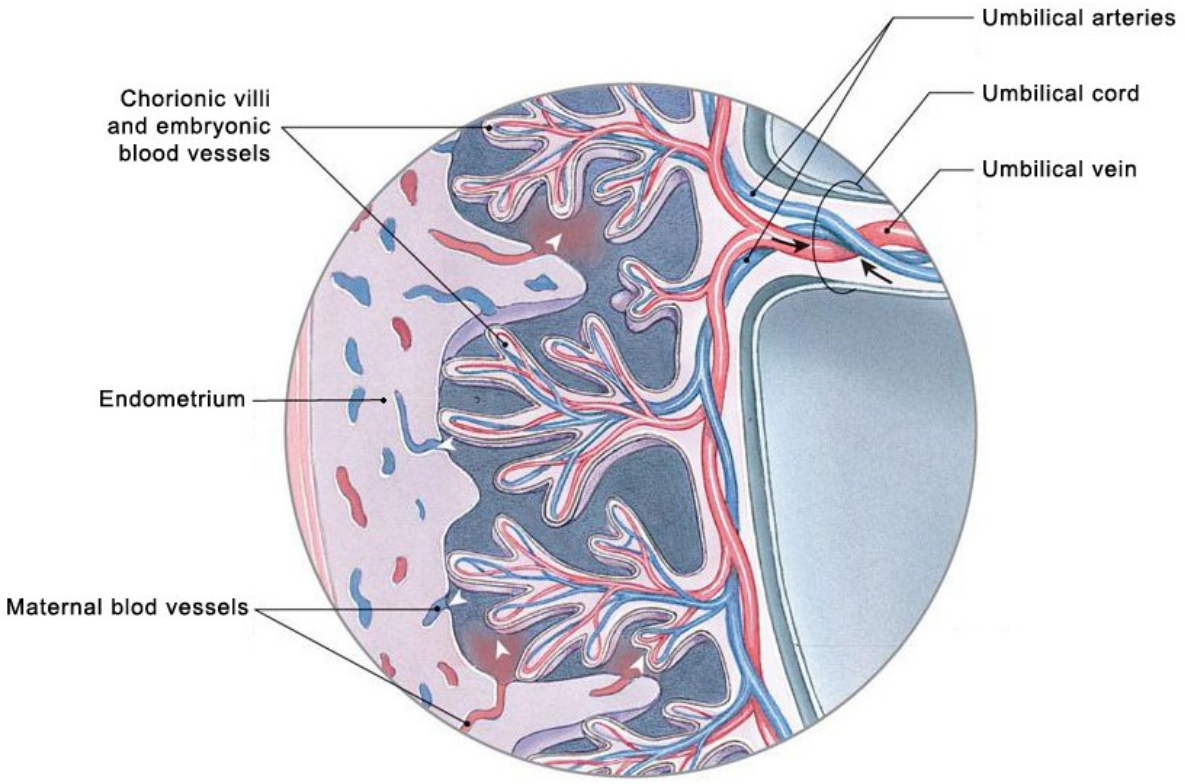

Fig. 2 Structure of the infiltrating chorionic villi. Digital structure composed by embryonic vessels and trophoblast multilayer, in touch with the maternal endometrium. Chorionic villi permit the exchange of nutrient and waste between embryo and mother.

Furthermore, STB cells are involved in the exchange of nutrients and waste as well as the gas required for embryo respiration (Kaufmann et al., 2003). STBs are also important in pregnancy maintenance. The induction of hormones such as chorionic gonadotropin (hCG) and placental lactogen (hPL) enable this to take place (Graham et al., 1992).

The second mechanism involving CTB proliferation enables chorionic villi that attach to the uterine wall to be formed (Knofler, 2010; Red-Horse et al., 2004). These chorionic villi may generate structures called extravillous trophoblasts (EVTs) which migrate into the decidua. Some are known as interstitial trophoblasts (iEVTs) and migrate to the deep endometrial layer or even to the myometrium of the mother. Another subset of these cells, endovascular trophoblasts (enEVTs), acquires endothelial traits and forms the new blood vessels in the embryo, which replace previously disrupted uterine vessels. Thus, uterine arteries are remodelled into low-strength and high-capacity uterine placental arteries (Lyall, 2006).

Therefore, the non-proliferative cell layer, syncytiotrophoblast, needs to be constantly supplied from below by fusing CTB cells (Kar et al., 2007; Richart, 1961). It is interesting to note how this cell fusion occurs only between CTB and the overlaying syncytium and not between adjacent CTB cells.

2.2 Miscarriage and related risks

Pregnancy loss by natural causes within 20 weeks of gestation is called spontaneous abortion or miscarriage. After this point, such episodes are referred to as stillbirth. About 80% of abortions occur in the first trimester (12 weeks) of pregnancy. The spontaneous abortion rate fluctuates at around 10% for women under 35 years old and increases up to 50-74% for those over the age of 40 (de la Rochebrochard et al., 2002; Nybo et al., 2000). In most cases, miscarriage remains a unique event. However, sometimes (between 0.5 and 1% of cases) this event may occur three or more times consecutively. In this case, recurrent spontaneous abortions are referred to (Bulletti et al., 1996). Risk factors are numerous and range from chromosomal abnormalities (Vaiman, 2015) to autoimmune diseases or vertical pathogen transmission such as Cytomegalovirus (CMV) or chlamydia. Active and passive smoking (Sopori, 2002; Venners et al., 2004), drug and alcohol consumption are also known to increase risk of pregnancy loss (Ness et al., 1999). Other risk factors are obesity (Lashen et al., 2004) or diabetes mellitus but also previous miscarriages (Kashanian et al., 2006) as well as the parents' age (de la Rochebrochard et al., 2002; Maconochie et al., 2007; Nybo et al., 2000).

2.3 Curettage

The medical procedure of curettage (or dilatation and curettage), refers to the surgical removal of the embryo and part of the lining of the uterus by scraping and scooping. However, a non-invasive method exists, manual vacuum aspiration (suction curettage), which enables sedation and surgical complications to be avoided. The World Health Organization (WHO) recommends scraping curettage as a method of surgical abortion only when manual vacuum aspiration is unavailable. Curettage is the most common method of voluntary abortion. It is also carried out in case of miscarriage to resolve abnormal uterine bleeding and to remove an excess of uterine lining due to polycystic ovary syndrome or retained tissue as a result of missed/incomplete miscarriage (*The Johns Hopkins Manual of Gynecology and Obstetrics* Lippincott Williams & Wilkins, 2012).

2.4 Polyomaviruses

The Polyomaviridae family has existed since 2000, when the International Committee on Taxonomy of Viruses formally split the Papovaviridae family into two new families, Polyomaviridae and Papillomaviridae. The Polyomaviridae family includes Polyomaviruses (PyVs) (Delbue et al., 2012).

The human Polyomaviruses (hPyVs or simply PyVs) belong to Polyomaviridae family and to date 13 members that infect humans have been identified. However, there are some exceptions, such as the Simian Virus 40 (SV40) which is capable of infecting humans despite being not a hPyV (Barbanti-Brodano et al., 2004).

JC Polyomavirus (JCPyV) (Padgett et al., 1971) and BK Polyomavirus (BKPyV) (Gardner et al., 1971) were the first two Polyomaviruses isolated from humans. In 2007, another two viruses of this genera were isolated, the Washington University Polyomavirus (WUPyV) (Gaynor et al., 2007) and the Karolinska Institutet Polyomavirus (KIPyV) (Allander et al., 2007). Since then, the number of new Polyomaviruses which have been identified has increased more rapidly. To date, beyond the four previously mentioned PyVs, these have been identified: Merkel Cell Polyomavirus (MCPyV) (Feng et al., 2008), Human Polyomavirus 6 and 7 (HPyV6, HPyV7) (Schowalter et al., 2010), Trichodysplasia spinulosa-associated Polyomavirus (TSPyV) (van der Meijden et al., 2010), Human Polyomavirus 9 (HPyV9) (Scuda et al., 2011), Malawi Polyomavirus also known as Human Polyomavirus 10 (MWPyV, HPyV10) (Siebrasse et al., 2012), Human Polyomavirus 12 (HPyV12) (Korup et al., 2013), Saint Louis Polyomavirus (STLPyV) (Pastrana et al., 2013), New Jersey Polyomavirus 2013 or Human Polyomavirus 13 (NJPyV-2013, HPyV13) (Mishra et al., 2014).

Primary infection by PyV normally occurs asymptomatically in childhood. Subsequently, the virus remains latent in the human host. Polyomaviruses are ubiquitous or widespread throughout different human populations, but with differing infection rates. Viral reactivation may occur over an entire life span and temporary immuno-suppression or -depression may allow the development of various PyV-associated diseases. The oncogenic potential of Polyomaviruses, such as MCPyV that was isolated from the skin of a patient with Merkel cells carcinoma, have been shown in literature, demonstrating, in this case, its ability to cause cancer in human skin (Feng et al., 2008). Furthermore, more recently, JCPyV and BKPyV have been categorized by the International Agency for Research in Cancer (IARC) as “possible carcinogens” (WHO monograph 104, 2013) (Delbue et al., 2017).

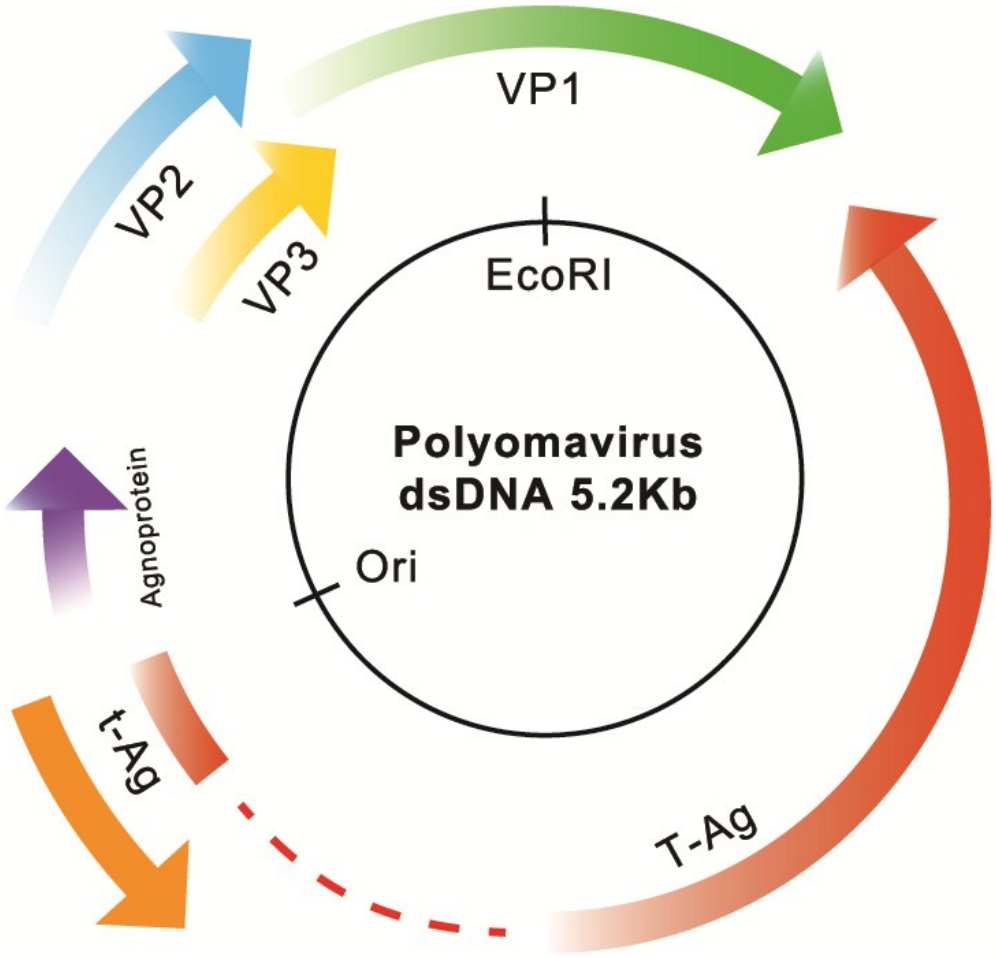

Fig. 3 Polyomavirus dsDNA genome. T-Ag and t-ag are the two early-synthetized proteins with oncogenic potential. VP1, 2 and 3 are the three late-synthetized structural proteins that constitute the viral capsid. Agnoprotein is the accessory protein. *Ori* is the bidirectional origin of replication of the polyomavirus genome.

Polyomaviruses are small, naked viruses with an icosahedral capsid containing a circular double-strand DNA. All PyV genomes, which are around five thousand base pairs in length, can be functionally divided into three distinct regions that encode for six major viral proteins. These are the early-, late- and non-coding control regions (NCCR). Bidirectional replication starts from the origin of replication (ORI) in the middle of the NCCR (Ferenczy et al., 2012) (Fig. 3).

Synthesis of the different early and late proteins occurs through the transcription of only two mRNA, subjected to alternative splicing. The early coding region is transcribed before DNA replication begins. Large Tumor antigen (T-Ag) and small tumor antigen (t-Ag) are encoded

by this region (Abend et al., 2009; Barbanti-Brodano et al., 2006). PyV tumor antigens are multifunctional regulatory proteins which are essential for replication since they drive the host cell towards the S phase and also initiate viral DNA. Later, the late coding region encodes the three viral structural proteins, VP1, VP2, and VP3, as well as the accessory agnoprotein. 360 molecules of the major capsid viral protein 1 (VP1) make up the PyV capsid, which contains 72 pentamers. Besides this, one minor capsid viral protein, VP2 or VP3, binds to each pentamer from the inner side of the capsid, so only VP1 is exposed on the surface (Ferenczy et al., 2012; Mazzoni et al., 2012).

Finally, the agnoprotein appears to be multifunctional, with roles such as regulating viral transcription or inhibiting host DNA. (Khalili et al., 2005). The similarity of T-Ag and VP1 sequences to the various Polyomaviruses is shown in Fig. 4a and Fig. 4b, respectively. Note that JCPyV and BKPyV T-Ag and VP1 are closely related to each other and to the SV40 sequences.

Polyomaviruses traces were identified in different biological specimens, for example urine, feces and serum. Therefore, these data suggest different ways of transmission, such as respiratory, oral-fecal, blood, and urinary routes, but the modalities of inter-human virus transmission are, as yet, not completely understood (Barbanti-Brodano et al., 1998).

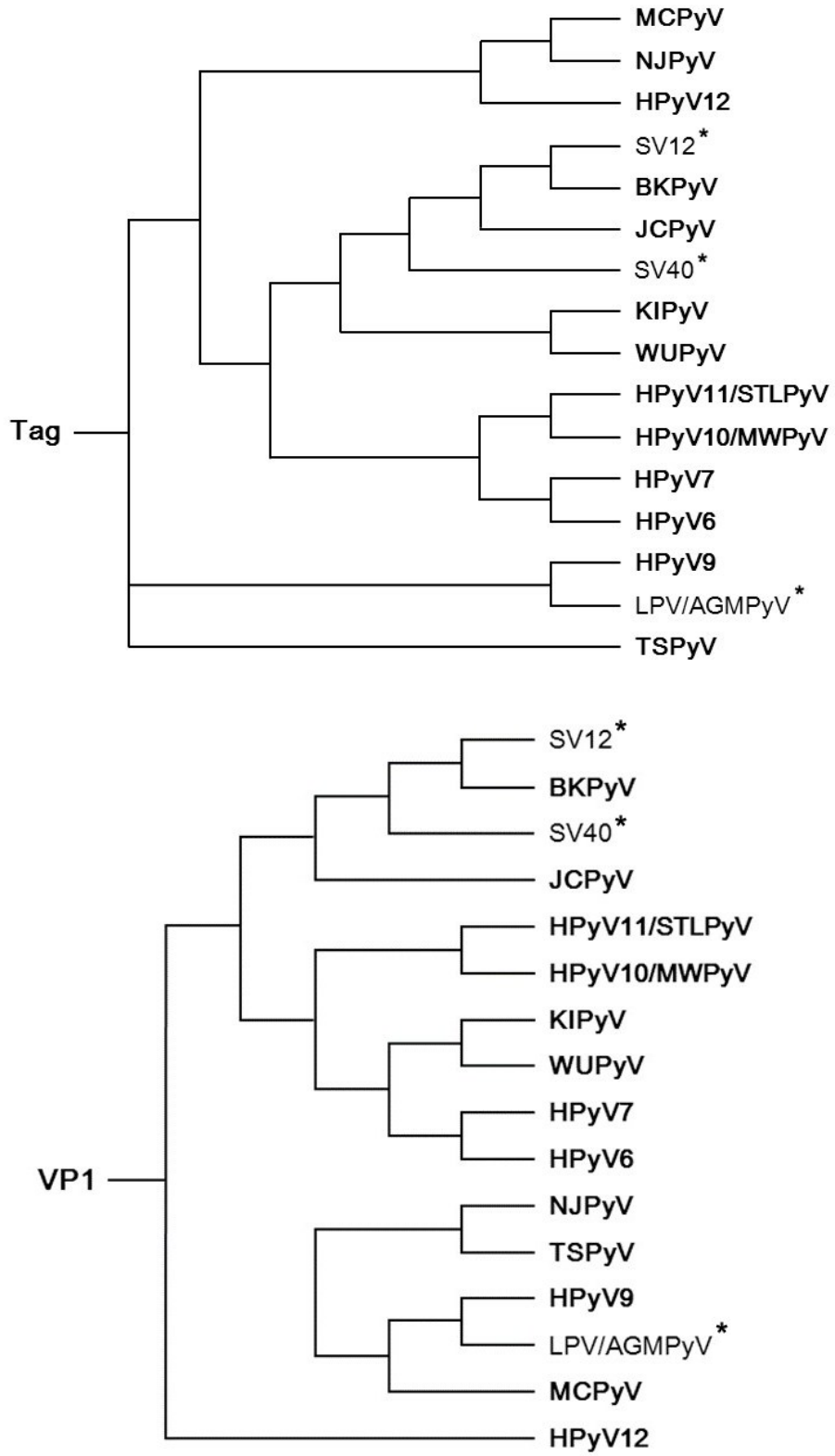

Fig. 4 The similarity of T-ag and VP1 sequences among different polyomaviruses is shown. Simian PyVs are indicated with * to the right of the name. (A) Note that JCPyV-Tag and BKPyV-Tag are more closely related to those of SV40, SV12 (simian agent virus 12) than to the MCPyV-Tag or other Tag of other polyomaviruses. (B) Note that JCPyV and BKPyV VP1 sequences are more closely related to those of SV40, SV12 than to the VP1 of other polyomaviruses like MCPyV.

2.5 Oncogenic potential

Cells can be permissive, semi-permissive or non-permissive to PyV infection. When a Polyomavirus enters a non-permissive cell, where viral genome replication is not supported, the infection is abortive and T-ag and t-Ag are the only genes expressed. Alterations to the normal cell cycle by T-ag/t-ag coupled with interaction between these oncoproteins and cell factors involved in the cell growth programme can cause cell proliferation and transformation (Barbanti-Brodano et al., 2004; Tognon et al., 2003).

T-Ag is a nuclear phosphoprotein made up of about 700 amino acids which is considered to be the main factor responsible for the JCPyV infection process. T-Ag induces viral replication and gene transcription in the late region. Large T antigen is able to recruit the host cell DNA-polymerase complex; it can bind the hypophosphorylated Retinoblastoma protein (pRb), a tumor suppressor protein, promoting oncogenesis and allowing premature release of the transcription factor E2F, which stimulates resting cells to enter the S-phase of the cell cycle (Eash et al., 2006). The C-terminal region of T-Ag includes the tumor suppressor protein 53-binding domain (p53). p53 is a tumor suppressor factor which is normally found in low concentrations in healthy cells. Following DNA damage or the presence of oncogenes, p53 production increases to activate the DNA repair mechanism and, if necessary, apoptosis and senescence mechanisms. T-Ag binding of this important protein does not allow cells to begin repairing DNA or apoptosis to take place but it does favour cell cycle progression. Thus, T-Ag changes the normal cell cycle, favouring cell multiplication and viral proliferation (Dilworth, 2002). Furthermore, T-Ag can bind other proteins, inducing tumor progression as, for example, β -catenin (Gan et al., 2004). T-Ag is also able to trans activate proto-oncogenes (c-myc, c-fos, c-jun) (Hollanderova, et al. 2003). Other factors, such as Insulin-like Growth Factor 1 (IGF-1), Platelet-Derived Growth Factor (PDGF) alfa and beta, Vascular Endothelial Growth Factor (VEGF) and Hepatocyte Growth Factor (HGF), are all activated through interaction with large T antigen (Ali et al., 2001; Cacciotti et al., 2001; Cacciotti et al., 2002).

2.6 JC Polyomavirus (JCPyV)

Humans are the natural host and reservoir for JC Polyomavirus (JCPyV), a virus which was isolated in 1971 from the brain of a Hodgkin lymphoma affected patient suffering from Progressive Multifocal Leukoencephalopathy (PML) (Padgett et al., 1971).

The JCPyV genome is about 5.130 base pairs (bp) long and is divided into two different functional regions, which are separated by the hypervariable non-coding control region. The first transcribed region is known as early region and is 2.4 Kb long. This region codes, among other elements, for large Tumor antigen (T-Ag) and small tumor antigen (t-Ag), which are both involved in viral replication and oncogenesis.

JCPyV genome late region, which measures 2.3 Kb, encodes for the structural Viral Proteins (VP1, VP2 and VP3) and for Agnoprotein. VPs are translated by a common mRNA through alternative splicing. JCPyV virion is a non-enveloped, icosahedral capsid, measuring about 45 nm in diameter. Its structure is mainly made up of VP1 (360 proteins each) and 30-60 copies of VP2 and VP3. In particular, VP1 is the exposed protein located on the surface of virions, so it is responsible both for contact with permissive cells and, therefore, antibodies (Imperiale, 2001).

JCPyV infection is enabled by binding which takes place between the viral VP1 and an N-linked glycoprotein with sialic acid in the permissive cell (Liu et al., 1998). When the virus has been endocyted into the host cell, it travels to the cell nucleus, where it is uncoated and starts transcription in the early region, T-Ag transcription, viral DNA replication and so on.

JCPyV is ubiquitous in the world population and initial infection typically occurs during childhood asymptomatically. Between 50 to 60% of children show specific antibodies against JCPyV, while among adults seroprevalence is around 40% (Elia et al., 2017; Kean et al., 2009).

JCPyV transmission routes have not yet been defined, but many studies show the presence of the Polyomavirus in different healthy human tissues, cells and fluids like urine, feces, sperm, brain, bone marrow, B-lymphocytes, kidneys, spleen, lymph nodes, and lungs.

Primary infection, such as for the other PyV, is followed by a lifelong, subclinical persistence of the viral genome. As a result of immune-suppression/-depression, JCPyV can reactivate and induce neoplastic development.

As already mentioned in the literature, it has been shown that PyVs are able to transform the host cell (Tognon et al., 2003). Firstly, it has been seen that JCPyV is able to induce different types of brain tumors when inoculated into animals. Secondly, after JCPyV was identified as the PML etiologic agent, many other cases correlating this virus and other brain tumors were

published (Brassesco et al., 2013; White et al., 2005). For example, interaction between JCPyV t-Ag and the IRS-1 or β chain has been shown to be a key factor for malignant medulloblastoma in children (Khalili et al., 2003).
Recently a correlation between JC Polyomavirus and human colorectal cancer has also been demonstrated. As already mentioned, JCPyV is commonly excreted in urine, both by immunocompetent and immunosuppressed subjects. This has also been shown with JCPyV being detected in wastewater in Italy and around the world (Bofill-Mas et al., 2010; Rossi et al., 2007). Therefore, it is easy to understand how ingesting food or water contaminated by JCPyV can easily lead to gastrointestinal tract infections. Other gastrointestinal tumors related to JCPyV and documented in the literature, include esophageal carcinoma (Del Valle et al., 2005), gastric carcinoma (Murai et al., 2007) and colorectal adenocarcinoma (Hori et al., 2005; Vilkin et al., 2012).

2.7 BK Polyomavirus (BKPyV)

The icosahedral capsid of the BKPyV virion is about 45 nm in diameter. It is made up of 72 capsomeres of pentameric VP1 and one V2 or VP3 on its inner side. The capsid contains a 5 Kb long double stranded circular-DNA. As with other PyVs, the BKPyV genome can be functionally divided into three regions: the early region, the late region and the NCCR. The early region encodes for small and large T-Ag, which are the major regulatory proteins in the virus. In particular, T-Ag is responsible for replicating BKPyV and also for the oncogenic potential of the virus. Both the structural proteins VP1, VP2, VP3 and Agnoprotein are encoded by the BKPyV genome late region. In contrast to VP2 and VP3, VP1 and Agnoprotein are translated within a different reading frame. However, the functions of Agnoprotein during infection are still hypothetical (Cioni et al., 2013; Gerits et al., 2012). NCCR directs early and late region transcriptions but sometimes can suffer deletions, insertions or duplications. Literature suggests that the resultant “rearranged NCCR” could be the product of high-level viral replications and could hypothetically enhance correlated disease progression (Gosert et al., 2008). Strains with rearranged NCCRs have been found in samples such as urine, plasma and tissue biopsy’ samples from patients with BKPyV-correlated diseases (Olsen et al., 2006; Randhawa et al., 2003).
Transmission routes are still unknown, but it is possible to infer that the major ones are oral and respiratory (Hirsch et al., 2003). As with the other PyVs, BKPyV infection occurs at a young age but the virus persists in lifelong latency in the kidneys and urinary tract (Minor et al., 2003).

BKPyV seroprevalence in healthy subjects is about 70-80% (Kling et al., 2012; Pietrobon et al., 2017a; Pietrobon et al., 2017b). Positive percentages grow up until 40-50 years old and then decrease slightly (Kean et al., 2009; Pietrobon et al., 2017a; Pietrobon et al., 2017b).
Different diseases seem to be linked with BKPyV infection. Two of the most studied diseases are haemorrhagic cystitis which affects allogenic hematopoietic stem cell transplant (HSCT) patients (Azzi et al., 1999; Hirsch et al., 2003) and nephropathy in patients subject to kidney transplants (Hirsch et al., 2002). Encephalitis and meningoencephalitis, pneumonia, and insurgence of bladder and prostate tumors have also been associated with BKPyV (Keller et al., 2015; Nickeleit et al., 2000). In particular, there are some case reports of central nervous system diseases (CNS) BKPyV infection-correlated in immunocompromised patients (Behre et al., 2008; Bratt et al., 1999). There are also case reports demonstrating PML like disease caused by BKPyV rather than JCPyV (Daveson et al., 2013). Among the cancer cases, there is a report of BKPyV antibody-positivity in a patient with bladder cancer (van Aalderen et al., 2013). Furthermore, a recent study demonstrates significantly higher seroreactivity for BKPyV (and MCPyV) in cancer patients demonstrating an association (Robles et al., 2013). Thus, it is clear that the patient's immune system being compromised due to organ transplantation or disease is the main cause of BKPyV, and more generally to Polyomaviruses, being reactivated. This can then lead to cancer (Binet et al., 1999; Gorrill et al., 2006).

2.8 Merkel Cell Polyomavirus (MCPyV)

Merkel Cell Polyomavirus was discovered in 2008 during studies on Merkel Cell Carcinoma (MCC). MCC is a neuroendocrine skin carcinoma with a mortality of 46% (Lemos et al., 2010), while its incidence is estimated at about 2.4/million/year in Europe (Eisemann et al., 2014) and 3/million/year in United States (Agelli et al., 2003).
The onset of this tumor is linked to MCPyV genome integration in Merkel cells due to ultraviolet (UV) light exposure and consequent DNA mutation (Wong et al., 2015). MCPyV genome and viral T antigen proteins are detected in up to 80% of cases of MCC. T-ag expression is restricted to tumor cells and it has been shown that the virus genome is clonally integrated into the host cells genome (Amber et al., 2013; Feng et al., 2008). Furthermore, T-ag expression is required for MCC tumor cells to survive (Houben et al., 2010). Persistent expression of MCPyV oncoproteins in MCC tumors and other viral protein interference with the host immune system (Paulson et al., 2014) seems to allow the virus to evade immune responses and induce tumor development.

MCPyV sequences have been identified in 27% of human chronic lymphocytic leukemia (CLL) samples (Pantulu et al., 2010) and also in various tissues from patients affected by different types of tumors (Bialasiewicz et al., 2009; Loyo et al., 2010). Moreover, MCPyV sequences were reported in lymph nodes (Boldorini et al., 2014; Rotondo et al., 2017a; Shuda et al., 2009), buffy coats of blood donors (Pancaldi et al., 2011) and peripheral blood samples, as well as skin and gut tissues, in those without MCC (Feng et al., 2008; Mazzoni et al., 2017a). So it can be said that MCPyV is a ubiquitous Polyomavirus whose human IgG antibodies were also detected in 60% to 80% of healthy subjects. These results suggest that MCPyV infection is widespread in the general population (De Gascun et al., 2013; Pastrana et al., 2009; Tolstov et al., 2009; Touze et al., 2010).

As with other PyVs, MCPyV infects humans during childhood and once acquired stays in the skin, life-long (Chen et al., 2011). Many details of the MCPyV life cycle are yet to be understood, as much about the natural host cells of this virus is still unknown. In addition, MCPyV struggles to replicate in many cell lines, so elucidating the details of its life cycle is difficult. Despite this, a recent study has demonstrated that human dermal fibroblasts are able to support MCPyV infection as natural host cells (Liu et al., 2016).

2.9 Simian Virus 40 (SV40) and previous studies

In our laboratory, the presence of Polyomavirus DNA in healthy people and also in subjects with different kinds of tumors and diseases (Barbanti-Brodano et al., 2006; De Mattei et al., 1994) has been demonstrated using different methods. In particular, over time, we have thoroughly investigated the Simian Virus 40 (SV40). SV40 is a monkey Polyomavirus administered to human populations through inoculation by a contaminated anti-poliomyelitis vaccine in the 1960s. Many other groups have tried to identify SV40 DNA sequences in cancer samples and in samples collected from healthy subjects using the PCR method. Some studies have succeeded, whereas others have failed. Within these indistinctly defined circumstances, we have developed a new indirect enzyme-linked immunosorbent assay (ELISA) test with two synthetic peptides which mimic Simian Virus 40 capsid viral protein antigens. In this study, only the samples found positive for both peptides were used, that is, those considered SV40-positive. Therefore, as well as identifying viral sequences of SV40, JC, BK, and MC Polyomavirus in different kinds of samples, our laboratory has also been able to find IgG antibodies against SV40. This indirect ELISA was developed to detect specific antibodies against SV40 viral capsid proteins (VP 1, 2 and 3) in human sera. This test is specific for anti-SV40 human antibodies and there is no cross-reaction with the closely

related human JCPyV and BKPyV Polyomaviruses. An average prevalence of 18% was found using this new method on serum samples collected from 855 healthy blood donors from 18 to 65 years old (Corallini et al., 2012).

Another important step for measuring the spread of viral infections in the human population was made by developing synthetic mimotopes which represent, in this case, the SV40 T-Ag oncoprotein. The resulting data enabled a better understanding of the epidemiology of this Polyomavirus. Furthermore, it also threw light on correlations between SV40 and human tumors or other pathologies (Barbanti-Brodano et al., 2004; Mazzoni et al., 2014b). Indirect ELISA tests have led us to shed light on SV40 in healthy and elderly subjects and also in pregnant women. What has been seen is that the overall prevalence of reactivity to large T antigen peptides was 20% in healthy subjects from 18 to 65 years old (Tognon et al., 2016). The data suggest that with age, there is a gradual decline in seropositivity to SV40. More specifically, this datum is statistically significant only if we compare most younger subjects to older ones (aged 18–30, 28%; 51–65, 15%; $P < 0.05$). In view of this, our study was enlarged to include samples of elderly Italian people with ages ranging from 66 to 100 years old. Indeed, we found an overall seroprevalence of 24% (Mazzoni et al., 2017b). It is important to note that the subjects we studied were too old to have been infected by contaminated anti-polio vaccines and this suggests that SV40 has spread in humans independently of such vaccines. The presence of SV40 antibodies in subjects of different ages suggests that several transmission and spreading modes may exist for this small DNA virus.

Furthermore, the presence of antibodies against SV40 proteins were also checked for in pregnant women from 15 to 48 years old. Indirect ELISA tests indicated that SV40 T-Ag-positive serum was found in 17% of these women (Mazzoni et al., 2017a) with no significant difference compared to the control group. The indirect ELISA method enables antibodies against the Polyomavirus to be identified even when PCR methods fail (Comar et al., 2014).

Recently, IgG antibodies reacting to BKPyV mimotopes in the sera of healthy subjects have also been identified after a new specific indirect ELISA test was setup (Pietrobon et al., 2017b). Previous studies aiming to identify serum antibodies against BKPyV suffered cross-reactivity with other Polyomaviruses, such as JCPyV and SV40. Thus, a new immunological tool was required to check for the spread of BKPyV in the human population (Viscidi et al., 2003). This new method enabled us to highlight antibodies against BKPyV VP mimotopes in healthy subjects from 18 to 90 years old with a prevalence of 72%. Furthermore, the results show that seroprevalence increases with age until 50 years old (40-50 years old, 81%), when it then decreases, in elderly subjects (62-90 years old, 65%).

As previously indicated, Polyomaviruses seem to be correlated with several human tumors (Barbanti-Brodano et al., 2006; De Mattei et al., 1994) above all, SV40, which has been found to be associated with human tumors and cancers such as human malignant pleural mesothelioma (MPM), glioblastoma multiforme (GBM), uveal melanoma (UM), osteosarcoma (OS) and non-Hodgkin lymphoma (NHL).

GBM is a very aggressive tumor which is responsible for 2% of all cancer-related deaths. In addition, it is able to resist all conventional radio- and chemotherapies. The small DNA tumor virus SV40 has been found to be associated with GBM. Moreover, one of our previous studies indicated that in serum samples from patients affected by GBM, the prevalence of antibodies against SV40 VP mimotopes was 34%. This datum was statistically higher than in control groups (15%; $P<0.03$) (Mazzoni et al., 2014a). Thus, SV40, has been detected at high prevalence in GBM specimens and its sequences have also been revealed in others brain and bone tumours (Martini et al., 1996; Martini et al., 2002).

In the case of MPM, the main causative agent is considered to be the mineral asbestos. In addition to asbestos exposure, a genetic predisposition to asbestos carcinogenesis has also been suggested (Testa et al., 2011). But many studies have also found an association between this tumor and SV40 (Cristaudo et al., 2005). Our laboratory contributed to this point of view by testing sera from subjects affected by MPM using the indirect ELISA assay. A high prevalence of serum antibodies reacting to the SV40 viral protein, 26%, was found for this group while the control group was 15%, representing a statistically significant difference ($P=0.043$) (Mazzoni et al., 2012).

Many other studies have reported genetic alterations in UM, the most common intraocular tumor BAP1, for instance, is a gene encoding a deubiquitinant enzyme mutated in several UM cases and also in malignant pleural mesothelioma. Thus, we also studied the presence of anti-SV40 antibodies as was done for MPM since SV40 may be a risk factor for UM onset, alongside other oncogenic agents such as UV irradiation. Indeed, the overall prevalence in UM patients from our study was 33%, as compared to 15-17% in the two control groups ($P=0.004$ and $P=0.038$ respectively) (Bononi et al., 2014).

As with previously mentioned tumors, oncogenic viruses may be a risk factor associated with OS neoplasia onset and progression in ways which are, as yet, to be elucidated. In literature, some studies have not reported SV40 in OS but our study found a high prevalence (44%) of antibodies against SV40 VP mimotopes and a statistically significant difference with the control group ($P<0.001$) (Mazzoni et al., 2015)

Furthermore, the aetiology of the most common lymphatic cancer, non-Hodgkin's lymphoma, is still poorly understood. SV40 is considered an oncogenic agent for NHL development. In

serum samples from NHL patients, antibodies both against SV40-VP and, more recently, against T-Ag mimotopes were found with a prevalence of about 42% and 37%, respectively. The difference between NHL and control prevalences is statistically significant (Mazzoni et al., 2017c; Tognon et al., 2015).

On the other hand, other possible associations between SV40 and other human pathologies have been shown to be negative with our indirect ELISA. For example, the prevalence of the two anti-SV40 VP mimotopes antibodies in patients with breast cancer was 15% as compared to 18% in the control group of blood donor women (Martini et al., 2013). Our serologic data also showed no association between undifferentiated nasopharyngeal carcinoma and SV40 infection. Indeed, immunologic data indicate that the prevalence of SV40 antibodies was 25% in those sera, whereas in controls it was 16%, but this difference was not statistically significant ($P > 0.05$) (Mazzoni et al., 2016). Samples collected from patients with these two types of SV40 non-associated cancers were usually used as second control groups (in addition to healthy subjects) during consecutive studies of association.

Work on the Merkel cells Polyomavirus (MCPyV) was also carried out by our laboratory. Possible associations between tumors and cancers, other than the Merkel cells carcinoma (MCC) are now being studied. Interestingly, a study of three MCC cases whose patients were treated with biologic drugs, mainly anti-TNF, was recently published by us. Two of the patients were affected by rheumatoid arthritis and the other by ankylosing spondylitis. MCPyV DNA sequences were detected in all three tissue samples but not in corresponding peripheral blood mononuclear cells (PBMC) obtained with patient blood samples. Direct ELISA testing found IgG antibodies against T-ag and small tumor antigen (t-ag) in 2/3 sera samples, and IgG antibodies against viral protein 1 in all of them. MCC tissues also resulted positive for MCPyV LT-antigen in immunohistochemical analysis.

Studies on JC Polyomavirus were also undertaken, mainly in correlation to male and couple infertility (Comar et al., 2012; Rotondo et al., 2012) since, as indicated above, JCPyV DNA sequences have also been detected in human sperm fluids, suggesting transmission could also occur sexually (Comar et al., 2012). Worldwide distribution of JCPyV genotypes is related to specific geographical regions. This is due to the fact that the first JCPyV strain infecting a male is then maintained for a lifetime, despite migration (Shackelton et al., 2006). This JCPyV genotype geographical specificity could be used as a marker (Yogo et al., 2004; Rotondo et al., 2017b).

Thus, in a recent study, mapping the geographic origin of males by genotyping JC Polyomavirus in sperm samples was carried out by us. The data obtained, in agreement with the previous hypothesis, confirm that it is indeed possible to trace male migration by studying

JCPyV genotypes, but also highlight that about in 10% of cases, a second JCPyV strain can be acquired by the individuals in adult age. In addition, this suggests that temporary windows of immunodepression or immunesuppression could expose individuals to a new viral infection of a different JCPyV strain (Agostini et al., 1996).

2.10 Human Papillomavirus (HPV)

Human Papillomavirus is a small double-stranded (ds) DNA virus belonging to the Papillomaviridae family. The Papillomaviridae family is divided into 16 genres (de Villiers et al., 2004), five of which belong to the human Papillomavirus category. Approximately 40 HPV types show tropism for the ano-genital area. These HPVs are transmitted with sexual encounters and world prevalence is 11.7% in women of reproductive age (Bruni et al., 2010). Some HPV cause genital warts, while others produce persistent infections that may evolve into carcinomas, such as cervical intraepithelial neoplasia (CIN) (Schiffman et al., 2003). According to their ability to induce benign genital warts or CIN lesions and cervical squamous cell carcinoma (SCC), HPVs are divided into high- and low- oncogenic risk. High-risk types (mainly HPV 16/18) have been associated with cervical cancer (90%; (Munoz et al., 2003)), low-risk HPVs (mainly HPV 6/11) with genital warts (Crosbie et al., 2013). Normally, most infections are asymptomatic and a vaccine for types 6, 11, 16 and 18 is available (Cutts et al., 2007). HPV infection cannot be detected by blood test but only using PCR methods performed on cervical scraping samples after a positive Papanicolaou (PAP) test (Molijn et al., 2005).

Table1. Epidemiologic classification of High-, Low- and Probably High-risk HPVs

HPV Group	HPV type
High-Risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82
Low-Risk	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81
Probably High-Risk	26, 53, 66, 68, 73, 82

HPV virions are envelope-free but are composed of a protein capsid made up of 72 hexa- or pentameric units with a icosahedral structure. The HPV genome, a circular double stranded (ds)DNA of approximately 8.000 bp is found within the capsid (Narisawa-Saito et al., 2007). The genome is divided into three regions: Long Control Region, a non-coding region for replication control and encapsidation; Early region, containing early non-structural genes and Late region, which contains structural genes. Eight genes are coded, six of which are transcribed at the initial stages of the viral replication cycle (early region E1, E2, E4, E5, E6 and E7) and two in the late stage (late region: L1 and L2) (Fig. 5)(Bravo et al., 2007).

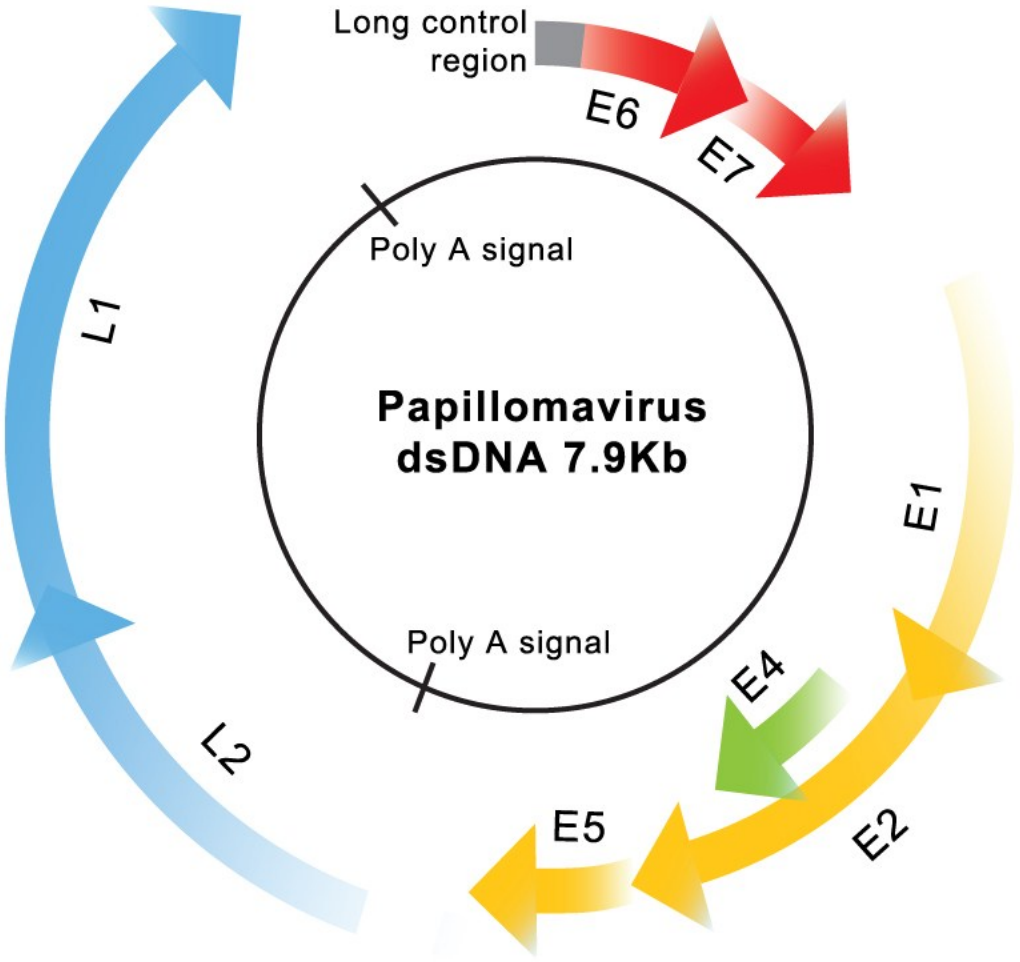

Fig. 5 Papillomavirus dsDNA genome. Early genes are indicated with the letter E (1,2,4,5,6 and 7). The genes coding for the two oncoproteins E6 and E7 are showed in red. Late transcribed genes, the structural ones, are indicated with the letter L (1 and 2). The Long control region is showed in grey. It is responsible for replication control and encapsidation.

Both genes and their respective encoded proteins have the same name. As already mentioned, the two structural proteins coded by the Late region are L1 and L2. L1 is the most important viral capsid protein forming the icosahedral capsid and is targeted by the antibody response. On the other hand, L2 is a highly variable protein that performs both structural and regulatory viral DNA encapsidation functions.

However, the first six proteins translated during HPV infection are regulatory proteins that interact with the host genome to give way to viral replication. E1 and E2 are directly involved in DNA replication. E2 gene specifically encodes for multiple proteins that regulate replication by binding DNA host specific sites (Wilson et al., 2002). Low E2 levels induce Early region

transcription, but at high levels E2 acts as a repressor by holding the binding sites of cell transcription factors. Its function as a regulator of oncoprotein E6 and E7 levels is especially important. Indeed, loss of E2 functionality causes neoplastic transformation (Bravo et al., 2007).

E6 and E7 proteins are essential for neoplastic transformation since cell cycle regulation is interfered with. Indeed, E6 is capable of binding the tumor suppressor protein p53, causing its degradation (Scheffner et al., 1990; Werness et al., 1990). p53 plays an important role in cell cycle regulation, as it induces both apoptosis in the event of severe DNA damage and p21, a cyclin/cyclin-dependent kinase complex inhibitor that blocks the cell cycle in G1 phase activation of (Vogelstein et al., 2000). At the initial stages of infection, E7 performs several functions among which negatively regulating the cell cycle and immune response in the host. In addition, if deregulated, it may undertake oncogenic activity, as with E6 (Felsani et al., 2006). E7 is able to bind pRb, a tumor suppressor protein and other members of its family (Dyson et al., 1989). pRb is a nuclear phosphoprotein, which regulates cell growth by inhibiting cell cycle progression beyond G1 phase (Korenjak et al., 2005).

Alongside E6 and E7, E5 protein inhibits apoptosis and contributes to inducing cellular hyperplasia (Kabsch et al., 2002). In addition, it has been demonstrated that E5 may increase cancer cell infiltrative capacity in cervical cancer (Genther et al.,2003; Maufort et al., 2010).

2.11 Role of viral infections in miscarriage

Many infectious agents have been correlated positively to spontaneous abortion (Benedetto et al., 2004) and other problems related to pregnancy. Studies have shown that viral infections, rather than bacterial or protozoa infections, can account for up to 15% of miscarriages and up to 66% of late spontaneous abortions (Baud et al., 2008; Srinivas et al., 2006).

The herpesviridae family includes different viruses which can establish latent infections in the human host. At a later time, following even temporary immunodepression, these viruses can reactivate (Whitley et al., 2001). Two of the most widespread members of the herpesviridae family are the Human Herpes Virus (HHV) and Cytomegalovirus (CMV). Both infect cells of the nervous system: HHV establish latency in neuronal cells and can reactivate at labial or genital levels (Margolis et al., 2007); CMV is contracted at a young age and can infect myeloid cells before going into latency remaining in the host lifelong (Koch et al., 2006).

In a 2009 study, DNA sequences of Herpes simplex virus (HSV) -1 and -2 were identified in tissue samples from spontaneous abortion in a group of Greek women. Positive cases were about three times more than those in the control group with a valid statistical difference (Kapranos et al., 2009). Thus, it seems that HSV have a role in early spontaneous abortion. These data have been confirmed by another more recent study on five thousand Korean women. Of HSV-2 seropositive women, 38.8% had a miscarriage compared with 29.6% of the control group (Kim et al., 2012).

There are no recent studies that support CMV association with spontaneous abortion, but there are in-vitro studies in the literature that have shown that CMV infection can cause placental dysfunction. It has been demonstrated that CMV can induce an inflammatory response (for example increasing TNF-α level) that enhances apoptosis in trophoblast (Chou et al., 2006; Chou et al., 2002). However, further studies are required to elucidate the true role of CMV in adverse pregnancy outcomes.

Human immunodeficiency virus (HIV) is a retrovirus commonly transmitted through unprotected sexual encounters or with the exchange of syringes between drug addicts. In 2013, the World Health Organization estimated that 34 million humans had been infected by HIV. Studies show that HIV infection could cause a myriad of problems during pregnancy, including miscarriage. However, it has also been shown that anti-retrovirus therapy can reduce pregnancy issues (Zolopa et al., 2009). However, it is very important to know that HIV is associated with bacterial vaginosis (Ledru, et al. 1997) which has been linked with early pregnancy loss (Donders et al., 2009; Rocchetti et al., 2011; Tavo, 2013). Thus, the correlation between HIV infections and miscarriage could be revised.

Dengue viruses (DEN), belonging to the flaviviridae family, are transmitted via mosquito bites usually in tropical areas. Viruses like Zika and West Nile belong to the same family. Until 2010, despite numerous studies being carried out, it was unclear if DEN virus was associated with pregnancy loss (Pouliot et al., 2010). However, a recent study has shown that Dengue fever seems to be a risk factor for miscarriage (Tan et al., 2012).

Finally, Rubella is a virus that affects children at a very early age, often being almost asymptomatic. However, if infection is acquired during the first 16 weeks of pregnancy, it can result in miscarriage or severe fetal development defects (Banatvala et al., 2004).

Pregnancy is a sophisticated process that involves various types of cells and is regulated by various complex mechanisms that are not yet completely understood. Some of the more critical stages of pregnancy are the implantation of the embryo, formation and development of the placenta and blood vessel remodelling. If any of these processes have an abnormal course, it can lead to miscarriage (Ball et al., 2006; Michel et al., 1990). It is easy to

speculate that an infection may interfere with the process of pregnancy. Although, it is true that for many pathogens an association with spontaneous abortion has already been demonstrated, it is also true that, with some exceptions, the mechanisms by which abortion is induced are not yet known. With serious illnesses, such as Dengue fever and malaria or during HIV infection, the change in the woman's immune response can lead to spontaneous abortion rather than a vertical transmission. However, some pathogens, including Plasmodium and DEN virus, have been identified at fetal tissue as well as placenta level, demonstrating maternal-fetal transmission before abortion. In addition to horizontal transmission, Polyomaviruses are even able to transmit vertically. This has been shown by detecting viral genomic sequences in placenta but also in embryo and fetal samples (Boldorini et al., 2010). This underlines the existence of several abortion induction mechanisms. Examining embryonic/fetal tissues, obtained from mothers with one of the abovementioned diseases, is therefore crucial to clarify whether the transmission of the respective pathogens is possible and whether these infections may or may not have led to miscarriage (Giakoumelou et al., 2016). Thus, our study focused on HPV and Polyomaviruses infections.

2.12 PyVs and HPV involved in miscarriage

2.12.1 JCPyV

A 1998 study, carried out during aborted fetal autopsies (placenta, brain and kidney), aimed at identifying BKPyV and JCPyV genomic sequences by PCR (Pietropaolo et al., 1998). The samples were collected from terminations carried out between the 16th and 24th week due to encephalic or kidney malformations or 18 or 21 trisomy. None of the samples was positive for JCPyV DNA. Despite negativity, it is known that JCPyV is a ubiquitous virus in the population (De Gascun et al., 2013), associated with male infertility (Comar et al., 2012) whose presence has been demonstrated in the urine of pregnant women (Arthuret al., 1989; Flaegstad et al., 1991).

2.12.2 BKPyV

Some studies have suggested that BK Polyomavirus is able to cross the placenta and hypothetically infect the fetus, but in literature such prevalence is fluctuating. A study on 15 fetuses (Pietropaolo et al.,1998) aborted from the 16th to the 24th week of gestation (due to

early diagnoses of genetic defects or intrauterine malformations) were analyzed in PCR for Regulatory Region (RR) BKPyV DNA sequences. Results were positive for 12 placenta and 12 brain samples (80% each) and 9 kidney samples were also positive (60%). In addition, other important data emerged from the control group. In 12 placenta samples obtained from women who completed problem-free pregnancies, 50% showed BKPyV positivity (Pietropaolo et al., 1998). In a recent study, placenta and fetuses were analyzed from 5 samples obtained from premature spontaneous abortions (between 15 and 28 weeks) and 5 samples obtained from voluntary interruption due to early diagnosis of intrauterine malformations. The PCR amplified targets were two: VP1 and transcription control region (TCR). Analysing both target, a total 70% (7/10) of the fetal organ samples was positive for BKPyV DNA sequences. It is interesting to note that changing PCR target, number of positive samples differ. Only one placenta out of ten was positive to virus DNA sequences. However, upon studying the BKPyV genome in the infected placenta and in the corresponding infected fetus, it was noted that the DNA sequences belonged to two different subtypes of the virus (Boldorini et al., 2010).

Conversely in 2011, another study analyzed chorionic villi in 22 cases of early spontaneous abortion (in the first or second trimester of pregnancy), 25 cases of diffuse villitis and 102 cases of villitis of unknown aetiology (gestational age ranged from 30 to 42 weeks). In this work in-situ hybridization which reported total negativity for BKPyV was used in all analyzed samples, suggesting that this virus has no role in the pathogenesis of these pathologies (Cajaiba et al., 2011). However, only a few samples were collected for this study during the first trimester of pregnancy (the others should be called stillbirth); and all the other studies analyzed samples from the second/third trimester. In conclusion, further investigations are needed in order to discover whether the BK virus could be associated with miscarriage.

2.12.3 MCPyV

Studies have been carried out in order to investigate the presence of MCPyV sequences in the placenta of women subject to spontaneous abortion, intrauterine fetal deaths or voluntary terminations (as controls) between the 11th and 42nd week of gestation as well as on aborted embryo/fetuses (Sadeghi et al., 2010). The results showed that only one patient out of 289 was positive to MCPyV sequences, while the corresponding abortion fetus was negative, suggesting that no vertical transmission took place. In parallel, a screening performed on patient sera taken within the ninth week of gestation demonstrated the presence of IgG for MCPyV in about 46% of cases (212/462).

2.12.4 HPV

An association study of HPV infections and miscarriages in the first trimester of pregnancy showed that in 60% (15/25) of cases there was positivity for HPV DNA sequences (E6/E7 sequences) as compared to 20% (3/15) in controls (Hermonat et al., 1997). More specifically, the same miscarriage samples were able to demonstrate that the first target of HPV embryo infection was the syncytiotrophoblast, the external multi-nucleated layer which invades the uterus wall to establish nutrient circulation between the embryo and the woman (Hermonat et al., 1998).

Thus, HPV has been shown to replicate in trophoblastic cell lines suggesting potential vertical transmission of the infection (You et al., 2003; You et al., 2008). Transplacental transmission of HPV has also been demonstrated in a study comparing samples from the mother during pregnancy (genital tract scraping and peripheral blood) with newborn samples (oral, axillary and inguinal swab, nasopharyngeal aspirated and umbilical cord blood) and the placenta. The results showed placental infection in 23% of cases, with vertical transmission to the newborn in 12% of cases (Rombaldi et al., 2008).

However, recent studies on the effects of HPV infection upon miscarriage have shown contradictory results (Perino et al., 2011; Skoczynski et al., 2011; Yang et al., 2013). All of the already published studies were performed on no more than 150 women and none reported direct effects by the infection on pregnancy. Indeed, one of these studies showed a statistically significant association between HPV infection in the father and complications during the early stages of pregnancy. This work was carried out on samples collected from 199 Italian couples who required access to in-vitro fertilization (66.7% in HPV infected couples versus 15% of controls with no HPV infection, $P < 0.01$; (Perino et al., 2011)). Therefore, the latest data challenges the association between HPV infection and miscarriage without clarifying any effects including, among other possible causes, paternal positivity to the virus.

Therefore, there is still no evidence to point out the effects of HPV infection in early embryonic development. However, there are *in vitro* studies suggesting that this infection may lead to an increase in blastocyst cells apoptosis (Calinisan et al., 2002) and a lower capacity of penetration of the trophoblast in the maternal endometrium. In fact, a down-regulation of E-Cadherin has been demonstrated in trophoblast cells infected by HPV16 (Boulenouar et al., 2010).

3. AIM

The literature has shown that autoimmune diseases and vertical transmissions of pathogens are also risk factors for pregnancy. Many infectious agents are associated with spontaneous abortion and there are several studies which demonstrate that viral infections are responsible for about 15% of early abortions. Human Polyomaviruses, which are ubiquitous in the world population, have been identified in different biological specimens such as sera, urine and male semen. These data demonstrate different ways of inter-human transmissions including vertical transmission. This has been shown by detecting viral genomic sequences in placenta and fetus organ and blood samples. Furthermore, the ability of the Human Papillomavirus to replicate in trophoblastic cell lines has already been documented and thus, vertical transmission is conceivable.

This study was carried out since the scientific community has no general consensus on the matter and there is a lack of studies correlating the aforementioned viruses and miscarriages. Therefore, the aim of this study was to define whether an association exists between spontaneous early pregnancy losses and silent intracellular Polyomaviruses and Papillomaviruses infections. To this purpose, chorionic villi obtained from miscarriages, which occurred during the first trimester of pregnancy, were screened for the presence of JCPyV, BKPyV, MCPyV and HPV genomic sequences. To evaluate if infections were vertically transmitted from mother to embryo, samples of pregnant women's blood were collected. Then, peripheral blood mononuclear cells (PBMC) were separated from each whole blood sample to investigate for the presence of viral-DNA sequences. Chorionic villi and PBMC samples from women undergoing voluntary interruption of pregnancy, were also analyzed as a control group.

The objectives pursued in this study were:

1. To analyze for the presence of JCPyV, BKPyV, MCPyV and HPV at the level of chorionic villi with PCR, real-time PCR and Droplet Digital PCR methods. Collected tissue samples were then homogenized and treated for total DNA extraction;

2. To look for genomic sequences of JCPyV, BKPyV, MCPyV and HPV in total DNA extracted from corresponding PBMC samples from pregnant women;

3. To evaluate viral loads in chorionic villi and PBMC, in order to understand if viruses were in a latent or active replication phase. Furthermore, to identify previous maternal infections by studying JCPyV and BKPyV seroprevalence by indirect ELISA tests.

4. MATERIALS AND METHODS

4.1 Patients recruitment

Patients were recruited at the Arcispedale Sant'Anna of Cona (Ferrara, Italy) from the Gynecology and Obstetrics unit. All the samples for this study were collected there by Prof. R. Marci, Dr. R. Capucci and their collaborators sent to the Laboratory of Cellular Biology and Molecular Genetics of the Ferrara University for the analysis.

Chorionic villi samples were collected with curettage surgery from pregnant women subject to miscarriage (M samples n=98) and from women who had undergone voluntary interruption of pregnancy (V.I. samples n = 103).

The mean age of the patients was 33.1 years old with a median age of 34 (ranging from 15 to 48 years old). More specifically, women subject to miscarriage have a mean and median age respectively of 35 and 36 (ranging from 20-45 y/o), as compared to a mean age of 31.5 years old and a median of 31 for women undergoing voluntary terminations (ranging from 15-48 y/o).

Study participants provided written informed consent at the time of hospital admission. Specimens were collected and coded with a progressive number and indication of birth date only.

4.2 PBMC and serum separation from blood

Peripheral Blood Mononuclear Cell (PBMC) samples were obtained by density gradient centrifugation from pregnant women's blood samples using Histopaque-1077 (Sigma-Aldrich), a mixture of Ficoll, polysucrose and sodium diatrizoate. In particular, 92 M and 102 V.I. samples were collected. At the same time, the fraction of serum of each sample was also collected.

4.3 DNA extraction from chorionic villi tissues and PBMC samples

Total DNA from Chorionic Villi tissues was extracted using the DNeasy Blood and Tissue Kit commercial kit (Qiagen) while PBMC samples were extracted with the QIAmp DNA Blood Mini Kit (Qiagen), both according to the respective manufacturer's instructions.
Each one of the DNA extracted samples were quantified by spectrophotometric analysis (NanoDrop 2000, Thermo Scientific). DNA was stored at -80°C until the time of analysis. Furthermore, to test the suitability of the extracted DNA for PCR analysis, isolated DNA was PCR amplified with ß-globin primers (Pancaldi et al., 2009).

4.4 PCR detection of HPV DNA sequences

The PCR method was used in order to detect for HPV DNA sequences in the DNA samples. The amplified genomic region codified the viral major capsid protein L1. Sequences of the primers used for the amplifications are showed below:

GP5 5'-TTTGTTACTGTGGTAGATAC-3'

GP6 5'-GAAAAATAAACTGTAAATCA-3'

These primers enable HPV 6b, 11, 16, 18, 31 and 33 genotype detection. Each of the DNA samples was amplified, starting from 200ng of total DNA in a 25ul total reaction volume. Cycling conditions were: 94°C for 5 min; 45 cycles at 94°C for 1 min, 40°C for 2 min and 72°C for 1 min and 30 seconds; 72°C per 10 min (Martini et al., 2004). The amplified sequences were fragmented from 139 to 145 base pairs, depending on which type of HPV was detected. The HPV-16 complete genome cloned into pUC19 vector was used as a positive control (Bononi et al., 2012). Amplifications were performed with the SimpliAmp Thermal Cycler (Applied Biosystem).

Results were analyzed using gel electrophoresis (2.5% agarose gel) by staining with SYBR Safe DNA gel stain (Invitrogen), which is a substitute of ethidium bromide.

4.5 PCR detection of PyV DNA sequences

PyV DNA sequences identified in DNA extracted from PBMC samples were first verified with qualitative PCR. Every sample was tested twice in two different experiments using 500ng each. Amplifications were executed using the SimpliAmp Thermal Cycler. The amplified targets were VP1 DNA sequences for JCPyV and T-Ag DNA sequences for BKPyV and MCPyV.

Primer sequences used for identifying JCPyV DNA are listed below:

JC_VP1_Seq_F	5' – ACAGTGTGGCCAGAATTCCACTAC – 3'
JC_VP1_Seq_R	5' – TAAAGCCTCCCCCCCAACAGAAA – 3'

PCR amplification was performed as follows: 95°C for 10min; 45 cycles at 95°C for 30sec, 63°C for 40sec and 72°C for 1 min; 72°C for 10 min.

The following primers were used for amplifying the MCPyV sequences:

MCPyV-LT1709F	5' – CAGGCATGCCTGTGAATTAGGATG – 3'
MCPyV-LT1846R	5' – TCAGGCATCTTATTCACTCC – 3'

Cycling conditions were: 94°C for 5min; 45 cycles at 94°C for 30sec, 53°C for 30sec and 72°C for 45sec; 72°C for 10min.

BKPyV was identified with a Nested PCR. External-primers for the first amplification are listed below:

BB4-BK	5' – TTTTGGAACCTGGAGTAGCTCAGA – 3'
BB5-BK	5' – GCTTGACTAAGAAACTGGTGTAGATCAG – 3'

The amplification protocol began with 94°C for 5min; 45 cycles at 94°C for 1min, 58°C for 1min and 72°C for 1min; and finally 72°C for 10min.

Next, a Nested PCR was performed with the same amplification protocol but different primers as below:

BB2	5' – TAGGTGCCAACCTATGGAACAGA – 3'
BB3	5' – GGAAAGTCTTTAGGGTCTTCTACC – 3'

4.6 Real Time PCR detection of PyV DNA sequences

The Real Time PCR (or quantitative PCR, qPCR) method was used to identify the three Polyomavirus (JCPyV, BKPyV and MCPyV) DNA sequences in the chorionic villi samples. qPCR were executed with TaqMan technology and the amplifications were executed with a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad).

The standard curve was obtained using three different plasmids containing the target sequences. pMITCR1A plasmid (9491 bp) was used to investigate for JCPyV containing the complete JCPyV genome. The BKPyV specific plasmid was pBR322_BKV, including the complete BK Polyomavirus (9494 bp) genome. Finally, the plasmid used for the MCPyV analysis was pUC57_MC1. Please note that this plasmid was 2899 bp long and included only the large T antigen viral region. Standard dilution series, from 10^9 to 10 copies, were prepared for each plasmid to be used as standard curves.
For the JCPyV and BKPyV analysis, amplification was performed at 95°C for 10 min and then 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min (McNees et al., 2005).
Two specific Gene Expression Assays (JCV_AT Assay; BKV_AT Assay) were used as primers and probes, which had been produced on request by Life Technologies. The assays were drawn on a low-homology trait of the T antigen-coding region to maximize reaction specificity for each Polyomavirus. The sequences were as follows:

JCV_AT_F	5’ – AGTGTTGGGATCCTGTGTTTTCA – 3’
JCV_AT_R	5’ – GTGGGATGAAGACCTGTTTTGC – 3’
JCV_AT Probe	FAM – CATCACTGGCAAACAT – NFQ
BKV_AT_F	5’ – AGTGTTGAGAATCTGCTGTTGCT – 3’
BKV_AT_R	5’ – GGAGTTCCTTTAATGAAAAATGGGATGAAG–3’
BKV_AT Probe	FAM – CATCACTGGCAAACAT – NFQ

MCPyV analyses were performed with separated primers and probe that targeted T-Ag. Cycling conditions were: 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 5 secs and 60°C for 1 min (Goh et al., 2009). Primers and probe are shown below:

RQMCPyV_LT_1F 5′ - CACAGCCAGAGCTCTTCCT-3′

RQMCPyV_LT_1R 5′ - TGGTGGTCTCCTCTCTGCTACTG-3’

RQMCPyV Probe FAM – TCCTTCTCAGCGTCCCAGGCTTCA–TAMRA

4.7 Digital PCR detection of PyV DNA sequences

Droplet digital PCR (ddPCR) was performed using the same Gene Expression Assays, primers and probe as were used for qPCR on a ddPCR platform (Bio-Rad) as described below to identify JCPyV, BKPyV and MCPyV DNA sequences. In addition, another Gene Expression Assay (targeting EIF2C1 human gene, Bio-Rad) was used as a human reference for amplification. This allowed us to quantify the amount of DNA used for each well more accurately. ddPCR is a next generation PCR method which has better analytical sensitivity than the more well-known qPCR. This sensitivity is due to its ability to read single nucleic acids at a single molecule level and to generate up to 20000 amplification events per well, a very large number of experimental replicas.

DNA samples and the amplification mix were added to the 96 well PCR plates (Bio-Rad) with a final volume of 22ul per well. The DNA-mix solutions were emulsionated by Auto DG (Bio-Rad), then sealed with pierceable foil heat seal (181404, Bio-Rad) using the PX1 Sealer and amplified using the SimpliAmp Thermal Cycler (Applied Biosystem). Cycling conditions followed the manufacturer’s instructions for Supermix for Probe (Bio-Rad). Amplifications of all three PyV-sequences were performed as follows: 95°C for 10 min; 40 cycles at 94°C for 30 secs and 60°C for 1 min; 98°C for 10 min. Immediately after amplification, samples were read using the QX200 Droplet Reader (Bio-Rad). The number of positive and negative amplification events, determined by fluorescence amplitude, was analyzed using QuantaSoft Bio-Rad software. Viral load values were then normalized to the number of the human reference gene copies.

4.8 ELISA testing for JCPyV and BKPyV IgG antibodies

Given the high homology between the different Polyomaviruses, indirect ELISA tests for JCPyV and BKPyV antibodies detection were developed and standardized in our laboratories to avoid cross-reactions between antibodies for different Polyomaviruses. ELISA plates were coated with specific synthetic peptides (mimotopes) corresponding to the JCPyV and BKPyV VP1, respectively. The indirect ELISA employed herein has been already published (Pietrobon et al., 2017b).

Briefly, a computer-assisted method was used to select two JCPyV peptides from the late viral region by comparing the VP1 protein from JCPyV with the VP1 proteins from BKPyV, SV40 and other less homologous Polyomaviruses.

The same method was employed to select the two specific BKPyV peptides by comparing their VP1 late viral region with the homologous JCPyV, SV40 and other less homologous Polyomaviruses VP1 regions.

JCPyV antibodies did not cross-react with the SV40 and BKPyV hyperimmune sera used as control. Likewise, the two peptides for BKPyV antibodies detection did not cross-react with the hyperimmune sera against SV40 and JCPyV.

The amino acid sequences of the four peptides, known as VP1-K and VP1-N (JCPyV), VP1-L and VP1-M (BKPyV), were as follows:

VP1-K NH2 – KSISISDTFESDSPNRD – COOH

VP1-N NH2 – LMNVHSNGQATHDNGAGK – COOH

VP1-L NH2 – LKLSAENDFSSDSPERK – COOH

VP1-M NH2 – MLNLHAGSQKVHEHGGGK – COOH

Sera samples from patients were used as the primary antibody (diluted 1:20) and were analyzed for IgG antibodies reacting to JC and BK Polyomavirus-VP1 peptides. The secondary antibody used was goat anti-human, (goat anti-rabbit for control samples), IgG heavy (H) and light (L) chain specific peroxidase-conjugate (Calbiochem-Merck, Germany) diluted 1:10000 in Low Cross-Buffer (Candor Bioscience, Germany). Subsequently, 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution (Sigma-Aldrich, Milan), which reacts with the peroxidase enzyme, was used to yield the colour reaction. 96 wells plates were read on a spectrophotometer (Sunrise, Tecan) at a wavelength of 405 nm. The color intensity in wells was determined by optical density (OD). The cut-off for each plate was determined using the OD of three negative samples used as negative control. The mean of these three OD values was added to their standard deviation which was multiplied three times (+3SD). Sera were tested in double for both the mimotopes of JCPyV and BKPyV. Samples were considered JCPyV- or BKPyV-positive when reacting to both peptides (K and N for JCPyV; L and M for BKPyV).

4.9 Statistical analysis

Prevalence was compared between groups using the two-tailed Fisher's exact test. The Paired Students' t test was employed to evaluate the statistical significance of the PyV-viral load differences between groups. All statistical analyses were carried out using Graph Pad Prism 6.0 software. Statistically significant findings were considered when $P < 0.05$.

5. RESULTS

5.1 PyV sequences in chorionic villi

In this study, tissues samples (n=48) were collected by curettage surgery of women subject to miscarriage (M), whereas other specimens (n=66) were from voluntary interruptions of pregnancy (V.I.). Samples, were mechanically homogenized, then, DNA was extracted using the QIAGEN kit.

First, DNA from both cohorts was investigated by qPCR for the three Polyomaviruses, i.e. JCPyV, BKPyV and MCPyV sequences. qPCR reactions were performed using assays for large T-Ag sequences in the three PyVs.

In M cases JCPyV-positive sample prevalence was 8.3% (4/48), whereas in the V.I. cohort only 1.5% (1/66) tested JCPyV-positive. None of the samples tested BKPyV- or MCPyV-positive.

ddPCR was used in order to assay the same samples more analytically. Moreover, the sample size was increased with additional chorionic villi DNA from 50 M and 37 V.I., respectively. The ddPCR method, which employs the same primers and probes as qPCR, allowed an increased number of positive samples to be found. Indeed, JCPyV DNA sequences were identified in 52% (51/98) of M samples whereas 59.2% (61/103) was the prevalence detected in V.I. cases. BKPyV was found in 11.2% (11/98) of M cases and in 11.7% (10/103) of V.I., that is control samples. MCPyV DNA sequences were identified in 4.1% (4/98) of M samples and in 4.9% (5/103) of V.I. samples (Table 2)

5.2 PyV sequences in PBMC

Tissues specimens were collected with pair blood samples from the same patients (27 from M and 60 from V.I. cases). PBMC were obtained by density gradient centrifugation. Samples were analyzed using qualitative PCR. JCPyV DNA sequences were investigated with primers which are specific for VP1 coding sequences, whereas primers for the detection of BKPyV and MCPyV DNA were specific for large T antigen coding sequences.

JCPyV DNA sequences were detected only in one out of 60 V.I. samples (1.7%). MCPyV DNA sequences were found in 11.1% (3/27) of M samples and 15% (9/60) in the V.I. control. None of the M and V.I. samples tested BKPyV-positive.

Subsequently, another 65 M and 42 V.I. PBMC samples were tested using ddPCR, which is more sensitive than qPCR. Primers and probe employed were the same for both methods.

Data obtained by ddPCR in investigated samples showed 12.3% (8/65) prevalence in the M cohort and 26.2% (11/42) prevalence in the V.I. cohort for JCPyV sequences. MCPyV DNA sequences were detected in 9.2% (6/65) of M samples and 11.9% (5/42) of V.I. samples, whereas none of the samples tested BKPyV-positive. PyV prevalence in PBMC obtained from the two methods has been merged in Table 2.

5.3 HPV sequences in chorionic villi and PBMC

DNA extracted from tissue samples (98 M and 103 V.I.) and from paired blood samples from the same patients (92 from M and 102 from V.I. cases) were also tested by qualitative PCR for HPV DNA sequences. The PCR target was the gene encoding the HPV capsid protein L1. The resulting amplicons were migrated by gel electrophoresis.

PCR data indicated that HPV-positive samples had a 4.1% (4/98) prevalence and a 2.9% (3/103) prevalence in M and V.I., respectively (Table 2). None of the PBMC DNA tested positive for HPV L1-DNA sequences (Table 2).

Total HPV and PyV prevalence in chorionic villi and PBMC, from miscarriage and voluntary interruption cases, are showed below. Data obtained by different methods were merged.

Table 2. Prevalence of human Papillomavirus (HPV) and Polyomavirus (JCPyV, BKPyV and MCPyV) DNA sequences in chorionic villi and PBMC samples from M cases and V.I. controls

Cohort	**Prevalence of chorionic villi positive samples (number of positive samples/ samples analyzed)**			
	HPV	**JCPyV**	**BKPyV**	**MCPyV**
M	4.1% (4/98)	52.0% (51/98)	11.2% (11/98)	4.1% (4/98)
V.I.	2.9% (3/103)	59.2% (61/103)	11.7% (12/103)	4.9% (5/103)
P-value	0.716	0.323	1.000	1.000
Cohort	**Prevalence of PBMC positive samples (number of positive samples / samples analyzed)**			
	HPV	**JCPyV**	**BKPyV**	**MCPyV**
M	0% (0/92)	8.7% (8/92)	0% (0/92)	9.8% (9/92)
V.I.	0% (0/102)	11.8% (12/102)	0% (0/102)	13.7% (14/102)
P-value	1.000	0.637	1.000	0.506

M = Miscarriage; V.I. = Voluntary Interruption. Statistical analysis was performed using the two-tailed Fishers' exact test. The difference in prevalence for PyV and HPV sequences between M and V.I. samples was not statistically significant ($P > 0.05$).

5.4 PyV and HPV co-infections

Overall, 54/98 chorionic villi in the M samples tested positive for at least one PyV-genome sequence. Of these positive samples, 11/54 (20.4%) were positive for more than one PyV. PyV infections we found in 66/103 samples in the V.I. chorionic villi control group. Of these positive samples, DNA PyV co-infections were found in 12/66 (18.2%) (Table 3).

As we can see in more in detail in Table 4, 9/11 (81.8%) of M tissue samples are positive for both JCPyV and BKPyV DNA sequences. Co-infection by JCPyV and MCPyV happened only once. Same thing for co-infection by all the three Polyomaviruses together. As far as V.I. tissue samples are concerned, 10/12 (83.3%) samples were found to be co-infected by JCPyV and BKPyV. Otherwise, there are only two cases of JCPyV/MCPyV co-infection, while none result co-infected by three Polyomaviruses at the same time (Table 4). At trophoblast level, the results suggest some kind of correlation between BKPyV and JCPyV, in terms of enhanced proliferation. Indeed, of the 11 M samples which tested positive for BK Polyomavirus DNA sequences, 9 were co-infected by JCPyV (81.8%). In the V.I. cohort, this co-infection existed 10 times out of 12 (83.3%).

Only one case of voluntary interruption of pregnancy showed the presence of DNA sequences for both JCPyV and MCPyV when analysing PBMC maternal samples.

In addition, results showed HPV was identified with PyV (4/4 M; 3/3 V.I.) in all cases and controls. In particular, in the specimens of chorionic villi obtained from miscarriages, all four cases showed HPV and JCPyV co-infection. However, two of the HPV-positive control samples were co-infected by JCPyV and the other one by MCPyV (Table 4)

Table 3. General prevalence of PyVs and HPV co-infections in chorionic villi samples from M cases and V.I. controls.

	M	V.I.	P-value
Number of PyVs infected samples	55.1% (54/98)	64.1% (66/103)	0.200
Number of PyVs co-infections	20.4% (11/54)	18.2% (12/66)	0.818
Number of HPV infected samples	4.1% (4/98)	2.9% (3/103)	0.716
Number of HPV co-infections	100% (4/4)	100% (3/3)	1.000

M = Miscarriage; V.I. = Voluntary Interruption. Statistical analysis was performed using the two-tailed Fishers' exact test. The difference in prevalence of PyV and HPV sequences between M and V.I. samples was not statistically significant ($P > 0.05$).

Table 4. Detailed prevalence of co-infections between PyVs and co-infections between HPV and PyVs in chorionic villi samples from M cases and V.I. controls.

PyV co-infections	Prevalence of co-infection (Number of co-infected samples / total PyV-positive samples)		
	M	**V.I.**	**P-value**
JCPyV + BKPyV	81.8% (9/11)	83.3% (10/12)	1.000
JCPyV + MCPyV	9.1% (1/11)	16.7% (2/12)	1.000
JCPyV + BKPyV + MCPyV	9.1% (1/11)	0% (0/12)	0.478
HPV-PyV co-infections	**Prevalence of co-infection (Number of co-infected samples / total HPV-positive samples)**		
	M	**V.I.**	**P-value**
HPV + JCPyV	100% (4/4)	66.7% (2/3)	0.429
HPV + MCPyV	0% (0/4)	33.3% (1/3)	0.429

M = Miscarriage; V.I. = Voluntary Interruption. Statistical analysis was performed using the two-tailed Fishers' exact test. The difference in prevalence of PyV and HPV sequences between M and V.I. samples was not statistically significant ($P > 0.05$).

5.5 PyV viral loads

The viral loads shown in Figure 6 were obtained from the quantitative analysis on Polyomavirus DNA-sequences. Results are indicated in copies of viral genomes found in 10000 cells. Viral DNA copy numbers were found to be very low. It should be noted that PBMC samples are all BKPyV-negative. As we can see from Tables 5 and 6, there are no significant differences between cases and controls tissue samples and PBMC samples.

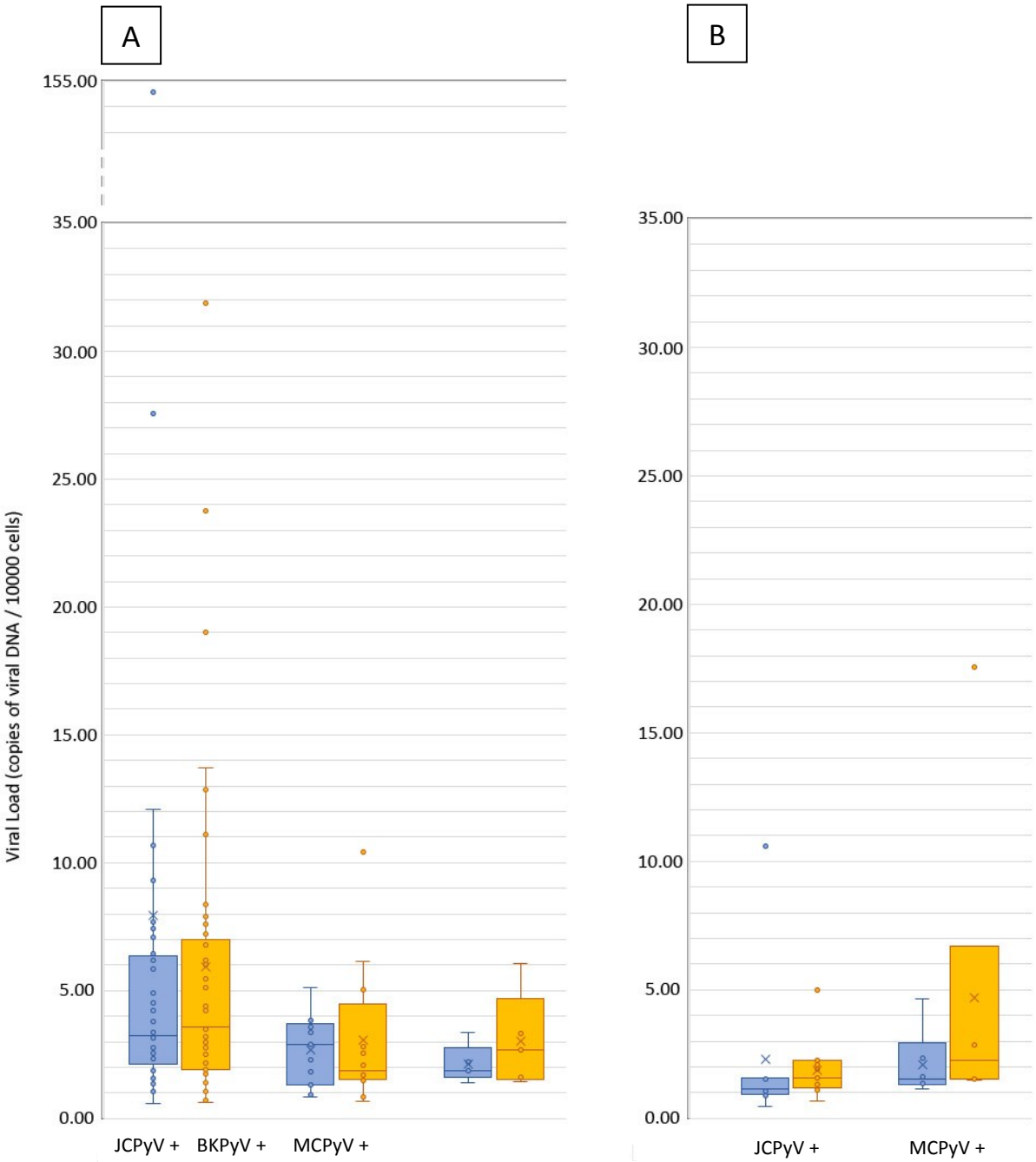

Fig. 6 JCPyV, BKPyV and MCPyV viral loads in positive chorionic villi and PBMC samples. Data from M cases is blue, data from V.I. controls is orange. (A) PyV-viral loads in chorionic villi; (B) PyV-viral loads in PBMC. The difference in viral load between M and V.I. samples was not statistically significant ($P > 0.05$).

Table 5. JCPyV, BKPyV and MCPyV viral load in chorionic villi samples from M cases and V.I. controls

PyV	Viral load (copies of viral DNA/10.000cells)				
	Mean	**Median**	**Range**	**SD**	**P-value**
JCPyV (M)	7.92	3.25	0.57 – 154.64	22.80	0.541
JCPyV (V.I.)	5.91	3.58	0.63 – 31.87	6.42	
BKPyV (M)	2.70	2.88	0.83 – 5.11	1.37	0.725
BKPyV (V.I.)	3.08	1.88	0.69 – 10.44	2.83	
MCPyV (M)	1.99	1.88	1.25 – 3.35	0.75	0.410
MCPyV (V.I.)	3.02	2.69	1.42 – 6.05	1.86	

M = Miscarriage; V.I. = Voluntary Interruption. Statistical analysis of the differences in mean value ± SD between groups was performed using the paired Students' *t* test. The difference in viral load between M and V.I. samples was not statistically significant ($P > 0.05$).

Table 6. JCPyV, BKPyV and MCPyV viral load in PBMC samples from M cases and V.I. controls

PyV	Viral load (copies of viral DNA/10.000cells)				
	Mean	**Median**	**Range**	**SD**	**P-value**
JCPyV (M)	2.29	1.13	0.46 – 10.57	3.37	0.675
JCPyV (V.I.)	1.88	1.56	0.66 – 5.00	1.15	
BKPyV (M)	0.00	0.00	0.00 – 0.00	0.00	---
BKPyV (V.I.)	0.00	0.00	0.00 – 0.00	0.00	
MCPyV (M)	2.09	1.52	1.15 – 4.66	1.32	0.267
MCPyV (V.I.)	4.70	2.26	1.50 – 17.55	6.33	

M = Miscarriage; V.I. = Voluntary Interruption. Statistical analysis of the differences in mean value ± SD between groups was performed using the paired Students' *t* test. The difference in viral load between M and V.I. samples was not statistically significant ($P > 0.05$).

5.6 JCPyV and BKPyV seroprevalence in samples from pregnant women

ELISA tests were performed on 89 M and 91 V.I. sera samples obtained from maternal blood drawings by density gradient centrifugation. Sera samples from M and V.I. patients were analyzed with two different indirect ELISA tests for IgG antibodies reacting to specific JCPyV and BKPyV VP1 mimotopes. ELISA plates were coated with two pairs of synthetic peptides corresponding to JCPyV VP1 (peptides VP1-K and VP1-N) and to BKPyV VP1 (peptides VP1-L and VP1-M).

In this study, sera were considered positive for one of the viruses when reacting with both mimotopes. Rabbit hyperimmune sera were used as a positive and negative control. JCPyV hyperimmune serum was used as a positive control and BKPyV hyperimmune serum was used as negative control for the JCPyV test. The same rabbit sera were used, in reverse, for the BKPyV test.

The cut-off value was in the range of 0.17-0.18 OD for both ELISA tests. Positive samples had an OD value over this cut-off and negative samples had an OD under this threshold limit.

The JC Polyomavirus seroprevalence in sera obtained from the women subject to miscarriage was 51.7% (46/89) while the prevalence in voluntary interruption samples was 49.5% (45/91) (Table 7). No statistically significant differences were highlighted with the Fisher exact test. When the data from BKPyV-positive sera were combined, the overall prevalence for M sera was 80.9% (72/91) while for the V.I. sera it was 80.2 (73/91). No statistical difference was found for these results, either (Table 8).

Table 7. JCPyV seroprevalence in sera samples from M cases and V.I. controls

Cohort	Positive samples / samples analyzed (%)		
	VP1-K	**VP1-N**	**VP1 (K + N)**
M	49/89 (55.1)	51/89 (57.3)	46/89 (51.7)
V.I.	49/91 (53.8)	50/91 (54.9)	45/91 (49.5)
P-value	0.882	0.766	0.768

M = Miscarriage; V.I. = Voluntary Interruption. Statistical analysis was performed using the two-tailed Fishers' exact test. The difference in prevalence of PyV and HPV sequences between M and V.I. samples was not statistically significant (P > 0.05).

Table 8. BKPyV seroprevalence in sera samples from M cases and V.I. controls

Cohort	Positive samples / samples analyzed (%)		
	VP1-L	**VP1-M**	**VP1 (L + M)**
M	80/89 (89.9)	75/89 (84.3)	72/89 (80.9)
V.I.	81/91 (89.0)	79/91 (86.8)	73/91 (80.2)
P-value	1.000	0.767	1.000

M = Miscarriage; V.I. = Voluntary Interruption. Statistical analysis was performed using the two-tailed Fishers' exact test. The difference in prevalence of PyV and HPV sequences between M and V.I. samples was not statistically significant (P > 0.05).

6. DISCUSSION

My experimental results indicate that the three different Polyomaviruses (JCPyV, BKPyV and MCPyV) have been identified in DNA from analyzed samples with a similar prevalence, both in M cases and V.I. controls. It turned out that the prevalence of HPV sequences in the same M and V.I. samples was also similar, while in PBMC DNA, HPV and BKPyV were not detected.

It is evident that no substantial differences exist between infections in miscarriages and infections in voluntary terminations. Differences between percentages are very low and have no statistical significance (P> 0.05). This is true both for surgical ablation tissues and PBMC from pregnant women's blood samples.

In almost all M and V.I. samples, there is no coincidence between chorionic villi specimen positivity for one of the viruses, and the corresponding maternal PBMC samples.

6.1 JCPyV infection in Chorionic villi and PBMC

JCPyV results are higher than the data found in literature, but it is important to note that different methods were used.

It is also of interest to note that JCPyV infection reached a high prevalence in DNA from trophoblast specimens. These data are innovative because previous published investigations did not report similar results when analyzing embryonal samples.

This study, carried out after almost a twenty year lack of new publications, using new methods and with a higher number of collected samples, allowed the presence of JCPyV DNA sequences to be identified even at low DNA viral load. Indeed, moving from a qPCR method to ddPCR increased almost tenfold the JCPyV samples which were found positive. The sensitivity increase is clear and implementation of the new method could explain the differences between literature and the present study.

Results suggest that JCPyV may have a tropism for the trophoblast, in addition to the well-established neurotropism. However, the low DNA viral load detected in the positive samples in this study indicate that JCPyV seems unable to replicate efficiently in trophoblast cells. It is possible that trophoblast could be one of the possible latency tissue for JCPyV. In this context, it can be speculated that JCPyV may remain in the latent phase in these cells and then could reactivate later during fetal development or after birth, when cells are more differentiated. This could have several potential consequences, as in some well-known pediatric tumors (i.e. medulloblastoma).

Finally, JCPyV DNA sequences were reported earlier in peripheral blood mononuclear cells, in particular in peripheral blood leucocytes and these data do not differ from results obtained in this study, which confirm previous results.

6.2 BKPyV infection in chorionic villi and PBMC

BKPyV sequences have been detected in placentas in some studies, whereas other groups did not reveal BKPyV footprints in similar samples. It should be noted that all previous reports were affected by the limitation of the small sample size. Nevertheless, literature indicates that BKPyV infection may occur in the embryo, and my results reinforce this assertion.

Interestingly, none of the PBMC samples obtained from pregnant women tested BKPyV-positive. At present, no studies suggest vertical transmission of BKPyV infection. Therefore, embryo infection does not seem to be directly caused by the woman.

6.3 MCPyV infection in chorionic villi and PBMC

Looking at previously published studies, I expected a very low number of MCPyV-positive trophoblast samples. Additionally, quantity and gestational periods of the samples used in this study were superimposable on those found in the literature. Nevertheless, higher prevalence in both cohorts was found.

On the other hand, results concerning MCPyV sequences in PBMC samples are in line with previous data from MCC remitted patients. Thus, data seem to fit with the profile of healthy subjects. Interestingly, 3/5 of the V.I. positive chorionic villi samples have correspondence with the pregnant women's PBMC results. This datum, found despite the small number of samples, may suggest that mother to embryo MCPyV-genome vertical transmission could be possible. This has never been shown.

6.4 HPV infection in chorionic villi and PBMC

The HPV prevalence in chorionic villi revealed in this study is of interest. HPV capacity to replicate in trophoblastic cell lines has been well studied, but prevalence in both cases and control groups of this study are lower than in those reported in others. Differences in results could be justified by the different target of PCR amplification. The genomic region amplified in the present study codified the viral major capsid protein L1, instead of E6 or E7 gene

sequences as in previous research. Obtaining different results using different HPV PCR targets has already been documented in literature.
However, completely HPV-negative PBMC results are in agreement with those reported in literature. In particular, it is possible to confirm the absence of HPV sequences in PBMC from healthy women.

6.5 Polyomavirus and Papillomavirus co-infections

Many studies have demonstrated that viral co-infections might occur. These investigations have reported multiple viral infections in the same family sets, in the same anatomic district, at the same time. Viral co-infections could be explained by a complementary effect among different viruses. For example, one virus could help another one by complementing a genetic function or helping to avoid pharmacological inhibition (Kamil et al., 2009). In these situations, wild-type or defective viruses could multiply in the infected host, thus inducing a specific associated pathology.
In other cases, co-infections could also lead to a boost in oncogenic activities. Indeed, several studies have documented that several factors are involved in the oncogenesis process. In this situation, oncogenic viruses should not be overlooked. This hypothesis was already postulated when, at the same time in different kinds of human tumors, footprints from BKV and SV40 (Martini et al., 1996), JCV and SV40 (Tognon et al., 2001) or HPV with HSV-2 and Epstein-Barr virus (EBV) (Gazzaniga et al., 1998) were detected. In particular, a study reported detecting both Papilloma- and Polyomavirus footprints in human genital tumors which suggests that these oncogenic viruses could interact enhancing their oncogenicity (Martini et al., 2004).
As said previously during the introduction, Polyomaviruses have been shown to be associated with neurological disorders and tumors in patients infected by HIV. Thus, the literature suggests that Polyomaviruses are associated with neurological disorders in AIDS patients.
At the same time, HPV and JCPyV or BKPyV co-infections in high-grade cervical lesions have also already been observed (Comar et al., 2011). In particular, the association between BKPyV and precancerous cervical lesions seems to suggest that this Polyomavirus participates with HPV-16 in the cell transformation process.
Overall, these data suggest that co-infections with small DNA tumor viruses may act synergistically in tumor onset/progression and prompted us to evaluate our results looking for possible Polyoma- and Papillomavirus co-infections. What we found is effectively interesting:

a high fraction of total infections resulted as co-infected. This could suggest some kind of interaction between different viruses of the same family. However, as there are no statistically significant differences between cases and controls, this prevalence does not appear to be associated with a higher chance of miscarriage.
In addition, it can be noted that the majority of co-infections includes JCPyV and BKPyV. Looking at this datum from another perspective, it can be inferred that, at trophoblast level, BKPyV needs JCPyV cooperation to enhance its proliferation.
Furthermore, results on HPV/PyV co-infections seem to be more interesting. Indeed, in 100% of HPV-positive samples there are also footprints of one of the three Polyomaviruses analyzed in this study. This is a result which has not, as yet, been reported in literature, suggesting close interaction between the two families of viruses.

6.6 Indirect ELISA testing for IgG against JCPyV and BKPyV

Indirect ELISA tests for IgG against JCPyV in M and V.I. sera, showed results comparable to the literature. Indeed, a very recent study on sera from healthy subjects with a similar mean age showed similar seroprevalence (11-30 y.o., 46%; 31-65 y.o., 54%) (Bononi et al., 2018). Once again, it can be noted that the patients' profile in this study corresponds to that in healthy subjects. Nevertheless, no significant statistical difference was highlighted between women subject to miscarriages and women who undertook voluntary interruptions.
On the other hand, data collected also for BKPyV seroprevalence, matches with the literature. Indeed, previous data obtained with the same method and patients' ages were not far from the mean age in this study.
Other immunological studies, which present different data, were mainly obtained using recombinant VP1 or other viral-like proteins as antigens. These proteins contain more epitopes in common with several PyVs, driving cross-reactivity between different anti-PyV antibodies and thus results with lower specificity than those found herein.

This study is innovative above all in terms of the type and number of samples analyzed. Indeed, previous published investigations have rarely examined large numbers of trophoblast samples. Furthermore, all the studies already published have shown at the same time results obtained from different types of samples and gestational times of termination are often different. In general, it is difficult to find results about chorionic villi, perhaps because of the difficulty in recovering this kind of specimen. Moreover, the samples were often obtained from patients with other pathologies that could affect results.

Finally, this study increases analysis sensitivity compared to literature, using new, innovative methods and homogeneous samples. Thanks to this, data partially present in the literature was confirmed, with more forcefully, with the addition of other new, interesting results.

7. CONCLUSIONS

PyV and HPV are both ubiquitous in the population with inter-human transmission. Thus, vertical transmission of these pathogens could rationally be a risk factor for pregnancy. Insufficient literature data on an association between Polyoma- and Papillomavirus and miscarriages prompted us to study these viruses in a little-studied sample type, i.e., chorionic villi. Furthermore, possible vertical virus transmission, from mother to embryo, was analyzed by collecting maternal PBMC. Knowing that viral infections are responsible for about 15% of total early pregnancy losses, some statistically significant differences between M and V.I. results were expected.

This study produced a lot of new data concerning the capability of PyV and HPV to infect chorionic villi and PBMC. Indeed, this is the largest investigation to date, based only on miscarriage trophoblast samples from first trimester of pregnancy. Furthermore, this is a massive study on HPV and four different Polyomavirus infections in parallel. These data allowed us to evaluate possible co-infections and the interaction between different viruses.

Nevertheless, the results indicate that JCPyV, BKPyV, MCPyV and HPV were identified in DNA from chorionic villi, but there were no statistical significant differences between M cases and V.I. controls. Thus, we cannot demonstrate any association between miscarriages and Polyoma- and Papillomavirus infections. Furthermore, the analysis of maternal PBMC samples showed DNA sequences for two Polyomaviruses, JCPyV and MCPyV, but all PBMC samples tested BKPyV- and HPV-negative. There are no statistical significant differences between the two cohorts.

JCPyV infection reaches a high prevalence in DNA from trophoblast specimens (M 52%, V.I. 59.2%). These are innovative data because previously published studies did not report similar results for embryonal samples. Previously divergent findings may be due in part to small sample sizes and in part to differences in techniques used for detecting viral footprints. Moreover, my results suggest that JCPyV may have tropism for the trophoblast. In addition, JCPyV sequences were confirmed in peripheral blood cells from healthy women.

The presence of BKPyV in trophoblast and placenta is still in discussion in literature. My BKPyV investigations show that this virus is able to infect chorionic villi with a prevalence of about 11% in M and V.I. samples. On the other hand, no BKPyV-positivity was found in PBMC samples.

Furthermore, the results of this study show that the Merkel cell Polyomavirus is able to infect both chorionic villi (M 4.1%, V.I. 4.9%) and PBMC (M 9.8%, V.I. 13.7%). It is interesting to note that in 3 V.I. cases, samples both from pregnant women and embryos tested MCPyV-

positive. Vertical transmission could be possible despite the small number of samples. This is a result which has never been reported in the literature.

From the analysis on HPV infections, the absence of its DNA sequences in the PBMC of healthy subjects and in pregnant women with miscarriage can be confirmed.

Interestingly, important innovative data was found concerning co-infections at trophoblast level. The results show that multiple PyV-infections are possible in this tissue and that these occur in about 20% of cases. Thus, it is possible that different Polyomaviruses cooperate to enhance proliferation. In particular, it seems that BKPyV needs the co-infection of JCPyV to replicate in chorionic villi. Actually, the DNA viral load results suggest that all the PyVs which were analyzed have problems proliferating in this kind of tissue. Thus, we could speculate that Polyomaviruses remain in these cells in the latent phase, possibly until late fetal development or after birth, when cells undergo more differentiation.

Meanwhile, results on HPV infections also show that this virus can co-infect chorionic villi with Polyomaviruses. Indeed, 100% of HPV-positive samples were also found to have PyV-genomic sequences. These results suggest a hard interaction between the two virus families, which has never been shown in literature. These data are very interesting due to their multiple implications. Last but not least, the possibility that some kind of viral infection combinations could be associated with miscarriage. For this reason, a future research drive in this direction would be interesting, perhaps also considering other viruses than Polyoma- and Papillomavirus.

Actually, in order to acquire more accurate and comparable data about HPV infections more sensitive methods should be used, such as, for the PyVs studied. Indeed, using qPCR or ddPCR for identifying HPV footprints could lead to higher prevalence being found. This could reduce the discrepancy with the literature or also highlight the presence of HPV DNA sequences in PBMC, which have never been documented before.

Furthermore, it would be interesting to combine the data of this study with the analysis of the paternal profile. In light of findings which indicate that JCPyV is able to infect also male semen, it could be possible that is the man to transmit the infection to the embryo. But for now, this is just a hypothesis.

In conclusion, results obtained with indirect ELISA testing show that JCPyV and BKPyV are highly ubiquitous in the population. Nevertheless, as anti-PyVs IgG were investigated and not IgM, we can say that these infections do not come into place during the gestational period. This is probably why low PBMC DNA prevalence was found. Therefore, even these data, when added to the previous shown in this study, draw profiles of healthy women who hypothetically pose no harm to their pregnancy. Unfortunately, there are no data from

MCPyV and HPV serological profiles to complete the picture. However, a new indirect ELISA test is now being refined in my laboratory to study MCPyV seroprevalence. This will potentially implement the study.

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