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Merkel cell Polyomavirus (MCPyV) in *non*-Merkel Cell Carcinoma (*non*-MCC)

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SUMMARY

ABBREVIATIONS

2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNP)
abhydrolyse domain containing 12 (ABHD12)
ADAM metallopeptidase domain 9 (ADAM9)
adaptor related protein complex 2 subunit A and M (AP2A1 and M1)
adenocarcinomas (AC)
ankyrin repeat domain 13A (ANKRD13Aa)
aryl hydrocarbon receptor interacting protein (AIP)
ataxia telangiectasia mutated (ATM kinase)
ATP binding cassette subfamily A Member 13 (ABCA13)
ATP binding cassette subfamily D member 13 (ABCD13)
ATP binding cassette subfamily D member 3 (ABCD3)
base-pairs (bp)
Basic Local Alignment Search Tool (BLAST)
BCL2 associated anthanogene 2, 3 and 5 (BAG2, 3 and 5)
bromodomain protein 4 (Brd4)
bromodomain protein 4 (Brd4).
cadherin 1 (CDH1)
cathepsin B (CTSB)
caveolae associated protein 1 (PTRF)
caveolae associated protein 2 (CAVIN2)
CCHC-type Zinc finger nucleic acid binding protein (CNBP)
Cdc20 homolog 1 (Cdh1)
cell division cycle 20 (CDC20)
cell surface glycoprotein 44 (CD44)
Chi- square ($\chi 2$)
chimpanzee polyomaviruses 2 and 3 (PtrovPyV 2 and 3)
Cutaneous B-cell lymphomas (CBCL)
Cutaneous T-cell lymphoma (CTCL)
Cystic Fibrosis (CF)
DEAD-box helicase (DDX24)
Delta-like protein 3 (DDL3)
detection of integrated papilloma sequences (DIPS)
EGF containing fibulin extracellular matrix protein 2 (EFEM2)
emerin (EMD)
epidermal growth factor receptor (EGFR)
eukaryotic translation initiation factor 4E binding protein (4E-BP1)
eukaryotic translation initiation factor 4E binding protein 1 (eIF-4EBP1)
family with sequence similarity 71 member E2 (FAM71E2)
F-box and WD repeat domain containing 7 (Fbxw7)

formalin-fixed paraffin-embedded tissue (FFPE)
four human Notch receptors (NOTCH1–4)
general transcription factor IIIC subunit 1 (GTF3C1)
gorilla polyomavirus 1 (GgorgPyV1)
heat shock protein 40 member C7 (DnaJC7)
heat shock protein 40 members A1 and B4 (DnaJA1 and B4)
heat shock protein 70 (HSPA1 and A4)
high density lipoprotein binding protein (HDLBP)
human polyomaviruses (HPyVs)
hypo-phosphorylated form eukaryotic initiation factor 4E (eIF4E)
inhibitor of nuclear factor kappa-B kinase-interacting protein (I⊚BIP)
inhibitor of κB (IκB)
insulin like growth factor 2 receptor (IGF2R)
interferon (IFN)
K homology domain-containing protein overexpressed in cancer (KOC=IMP3)
karyopherin subunit 2, 3 and 4 (KPNA2, 3 and 4)
large T antigen (LT)
large-cell NEC (LCNEC)
LT stabilization domain (LSD)
lysine-specific histone demethylase 1 (LSD1)
lysyl oxidase (LOX)
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)
MCPyV unique region (MUR)
mediator complex subunit 14 (MED14)
Merkel cells (MCs),
MicroRNA (miRNA)
microtubulin-associated protein 4 (MAP4)
mitochondrial carrier 2 (MTCH2)
myelin protein zero like 1 (MPZL1)
myoferlin (MYOF)
NF-kappaB (NF-kB)
NFκB essential modulator (NEMO)
N-myc downstream regulated gene-1 (NDRG1)
non-coding region (NCCR)
non-Merkel Cell Carcinoma (Non-MCC)
Notch 2 receptor (NOTCH2)
nuclear localization signal (NLS)
nuclear receptor binding SET domain protein1 (NSD1)
open reading frame (ALTO)
origin binding domain (OBD)
origin of replication (ORI)

Patched 1 (PTCH1)
PGAM family member 5, mitochondrial Ser/Thr protein phosphatase (PGAM5)
phosphatidyl-3-kinase/AKT/mammalian target of the rapamycin (PI3K/AKT/mTOR)
phosphatidylinositol-5-phosphate 4-kinase type 2 beta (PIP4K2)
pituitary tumor-transforming gene 1 protein-interacting protein (PTTPG1P)
platelet-derived growth factor receptor subunit (PDGFR)
pleomorphic carcinomas (PL)
PRA1 domain family member 2 (PRAF2)
progesterone receptor membrane component 2 (PGRMC2)
programmed death 1 (PD-1)
Programmed death ligand -1 (PD-L1)
prolyl 4-hydroxylase subunit alpha 3 (P4HA3)
prolyl 4-hydroxylase subunit P4HB)
proteasome 26S ATPase 2,3 and 4 (PSMC2, 3 and 4)
protein phosphatase 2 catalytic subunit (PPP2CA and CB)
protein phosphatase 2 regulatory subunit Aα and Aβ (PP2R1A and B)
protein phosphatase 2 scaffold subunit PP2AR1)
protein phosphatase 2A (PP2A)
protein phosphatase Mg2+/Mn2+ dependent 1A, 1B and 1G (PPM1A, B and G)
protein phosphatase regulatory subunit 1 (PP4R1)
Pseudomonas aeruginosa (*P. aeruginosa*)
pulmonary neuroendocrine carcinoma (NEC)
Quantitative real-time PCR (qPCR)
Rab18 (RAB18)
recitulon 4 (RTN4)
repeated measures analysis of variance (RM-ANOVA)
retinoblastoma protein (RB)
Retinoblastoma protein 1 (RB1)
Reverse transcription–PCR (RT-PCR)
Rhinovirus (HRV)
ribonuclease/angiogenin inhibitor 1 (RNH1)
secreted protein acidic and cysteine rich (SPARC)
ser/thr kinase 38 (STK38)
signal recognition particle 14 (SRP14)
signal recognition particle receptor subunit b (SRPRB)
small T antigen (sT)
sonic (SHH), Indian (IHH), and desert (DHH) hedgehog
Spearman's rho coefficient (r)
sphingosine-1-phosphate lyase 1 (SGPL1)
squamous cell carcinomas (SCCs)
Standard Deviation (±SD)
Staphylococcus aureus (*S. aureus*)

Statistical Package for Social Science (SPSS)
Stimulator of interferon genes (STING)
STIP1 homology and U-box containing protein 1 (STUB1)
sulfide quinone oxidoreductase (SQRDL)
surfeit 4 (SURF4)
T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3)
testis-specific Y-encoded-like protein 1 (TSPYL1)
the LT stabilizing domain (LSD)
the zinc-finger domain (ZN)
thioredoxin related transmembrane protein 3 (TMX3)
toll interacting protein (TOLLIP)
transcription elongation factor B subunit 1 (TCEB1)
transcription factor DP1 (TFDP1)
transcription factors E2F3 and 4 (E2F3 and 4)
translocase of inner mitochondrial membrane 8A (TIMM8A)
transmembrane protein 165 (TMEM165)
tripartite motif containing 38 (TRIM38)
truncated LT (tLT)
tubulin α1 (TUBA1B)
tubulin $\beta 2\alpha$ (TUBB2A)
Tumor (T)
type I IFN (IFN–I)
Ubiquitin-specific protease (USP7)
upregulated during skeletal muscle growth 5 (USMG5)
vacuolar protein sorting-associated protein 11 homolog (VSP11)
VAMP associated proteins A and B (VAPA and VAPB)
vascular endothelial growth factor receptor 3 (VEGFR-3)
virus-like particles (VLPs)
vitamin K epoxide reductase complex subunit 1 (VKORC1)
VPS39 subunit Of HOPS complex (Vam6p)
v-raf murine sarcoma viral oncogene homolog B1 (BRAF)

1. INTRODUCTION

1.1 Genome MCPyV

Merkel cell polyomavirus (MCPyV) is a naked double-stranded DNA virus belonging to the *Polyomaviridae* family [1]. Its circular genome of ~5.400 base-pairs (bp) encompassed three functional domains (**Figure 1**). The early region includes the "Tumor" (T) antigen gene locus [2], from which, alternatively-spliced RNA transcripts are produced. This region encodes for distinctive gene products: the large T (LT), small (sT), 57kT antigens and a product from an alternate frame of the LT open reading frame (ALTO) [3]. The LT, sT and 57 kT antigens, due to alternative splicing, share a 78 amino acid sequence at their N-terminal region [4].

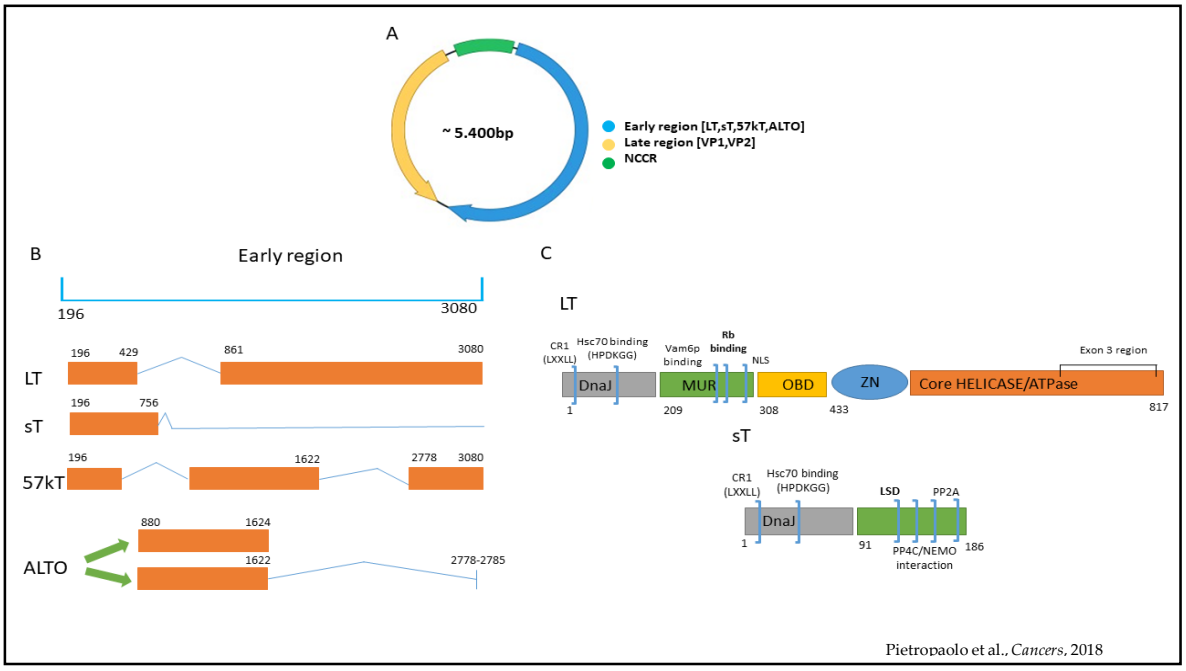


Figure 1. Structure of the MCPyV genome and the early region transcripts and the early proteins large T antigen (LT) and small T antigen (sT) with their functional domains. (A) Schematic presentation of the ~5,400 bp circular dsDNA genome that includes a non-coding region (NCCR), an early region encoding T antigens that coordinate viral replication, and a late region containing the genes for the viral capsid proteins VP1 and VP2. (B) Multiple transcripts are generated from the early region by alternative splicing, including LT, sT, 57 kT antigen (57 kT) and alternative frame of the large T open reading frame (ALTO). (C) LT contains the DnaJ domain with a conserved HPDKGG motif, the MCPyV unique region (MUR) with the retinoblastoma protein (RB) binding motif, the nuclear localization signal (NLS), the DNA or origin binding domain (OBD), the zinc-finger domain (ZN) and the helicase/ATPase domain. sT antigen encompasses the DnaJ domain, the LT stabilizing domain (LSD), and interaction domains for the protein phosphatases PP2A and PP4.

Similar to other human polyomaviruses (HPyVs), the MCPyV LT antigen contains a number of motifs and domains that play key roles in viral genome replication and transcription, as well as tumorigenesis (**Figure 1**). The N-terminal half encompasses the DnaJ domain, which consists of the CR1 motif (13–17 amino acids) followed by the HPDKGG, which sequence is responsible for Hsc70 binding [5,6]. The WXXWW sequence found in LT of other PyVs and that binds the mitotic checkpoint serine-threonine protein kinase Bub1 is absent in MCPyV LT [7]. At this position, MCPyV LT has a sequence known as MCPyV T antigen unique region (MUR), containing a binding motif for the vacuolar sorting protein Vam6p [8]. Adjacent to this, the conserved LXCXE retinoblastoma (RB) binding motif is present. Finally, a nuclear localization signal (NLS) with sequence RKRK is situated in the N-terminal region of LT [9]. The C-terminal region of LT contains an origin binding domain (OBD) and the helicase/ATPase domain [8]. Both the OBD and the helicase/ATPase domain are required for replication of the viral genome. The C-terminal region of LT of other HPyVs binds to p53, a tumor suppressor that regulates the gene expression in response to events such as DNA damage, leading to apoptosis, cell cycle arrest or senescence, and inhibition of angiogenesis, and is usually deregulated in cancer [10]. This p53 binding site is contained in the OBD and helicase/ATPase domain. The possible p53 binding domain in MCPyV LT and its interaction with p53 will be discussed below.

MCPyV-positive MCCs (hereafter referred to as VP-MCC) express a C-terminal truncated LT (tLT) due to nonsense mutations or frameshift mutations generating premature stop codons. Tumor-derived tLTs retain the DnaJ region and the RB binding domain, and sometimes the NLS, but lack the OBD and helicase/ATPase domain [5,11] (**Figure 1**). The C-terminal region contains several elements fundamental for viral replication, hence tLT fails to support viral replication [12]. As for other HPyVs, and in general for other tumor viruses, there is strong selective pressure within tumors to eliminate viral replication capacity [13].

MCPyV LT is rich in potential phosphor-acceptor sites (94 serine, 42 threonine, and 23 tyrosine residues). Li *et al*. found that phosphorylation of LT at S816 by ATM kinase induced apoptosis and thus contribute to anti-tumorigenic properties of the C-terminal domain [14]. Diaz *and colleagues* identified three additional phosphorylation sites: T271, T297 and T299. Mutation of T271 into alanine did not have an effect on viral replication. LT T297A stimulated replication, whereas LT T299A was unable to do so. The authors demonstrated that phosphorylation of T297 may negatively regulate viral replication by reducing the binding affinity of LT to the viral origin of replication (ORI), while T299 phosphorylation affects both binding to and unwinding of the DNA [15]. Taken together, truncation of the C-terminal region of LT and phosphorylation of specific residues in LT may abrogate viral replication. S220 is another phosphor-acceptor site and the effect of its phosphorylation will be discussed below. The phosphorylation status of LT in MCC has not been examined.

As a result of alternative splicing of a common precursor transcript, LT and sT share the 80 N-terminal amino acids [8]. The sT antigen contains the DnaJ domain but lacks the RB motif [16] (**Figure 1**). At its unique C-terminal region, sT encompasses two zinc-binding domains (CXCXXC motif), which provide structural and functional stabilities and two domains rich in cysteine and proline residues responsible for the sT interaction with protein phosphatase 2A (PP2A) (*see further*) [17]. A unique MCPyV sT domain, not present in sT of other HPyVs, is the LT stabilization domain (LSD) at residues 91-95. This region, as will be discussed later, is involved in inhibition of proteasomal degradation of LT (**Figure 1**) [18].

The late region encodes the major capsid protein VP1 and the minor capsid protein VP2 (**Figure 1**). MCPyV does not seem to express VP3 despite an in-frame internal start codon in the *VP2* gene [19]. When expressed in mammalian cells, VP1 (or VP1 and VP2) self-assemble into 45–55 nm diameter virus-like particles (VLPs) that are used in serological assays [20].

Interspersed between the early and late region is the non-coding control region (NCCR), which contains the ORI characterized by a core of 71-bp sufficient to initiate DNA replication (**Figure 1**). This core region consists of an AT rich tract and eight 5′-GAGGC-3′ LT binding motifs [12]. The NCRR also contains regulatory elements and bidirectional transcriptional promoters required for early and late viral gene expression [21]. The NCCRs of HPyVs such as BKPyV and JCPyV show often rearrangements that affect viral DNA replication, promoter activity, virus production and could help to increase the pathogenic properties of these viruses [22-24]. MCPyV NCCR polymorphism is found, but no specific NCCR architecture seems to be associated with VP-MCC as MCPyV variants with identical NCCR have been isolated from both MCC and *non*-MCC material [25]. However, MCPyV NCCR variation affects early and late promoter activities in a VN-MCC cell line and in human dermal fibroblast and wild-type LT inhibited both early and late promoter activities in both cell lines, whereas tLT had the opposite effect [25]. A recent study demonstrated the onset of insertions and deletions in the NCCR among an HIV-1-positive population [26]. Whether NCCR variation has an influence on viral replication and pathogenic properties of the virus remains to be investigated.

The molecular characterization of viral genomes has been useful to describe viral lineages associated with specific human populations, as demonstrated for other PyVs [27-29]. Phylogenetic analysis, performed on LT and sT antigens and on VP1, showed that MCPyV sequences circulate in Europe/North America, Africa, Asia, South America and Oceania groups, suggesting the occurrence of a viral divergence followed human migrations around the globe [30]. There is a significant evidence for an ancient and relatively stable association of PyVs with their hosts, suggesting that co-divergence is the main factor during the evolution [31]. However, deviations from co-divergence indicate that additional evolutionary processes are at play. Phylogenetic analysis, about the evolutionary history of MCPyV, showed that the MCPyV LT is most similar to gorilla polyomavirus 1 (GgorgPyV1) and chimpanzee polyomaviruses 2 and 3 (PtrovPyV 2 and 3) [1], raising the possibility that MCPyV

stems from a nonhuman primate (including chimpanzees and gorillas) and even ape-specific group of PyVs [31]. Nonhuman primates still represent an important proportion of the bush meat consumed in West and Central Africa (ca. 12%). Hunting and butchering of bush meat provide the major routes of pathogen and a cross-species transmission events (e.g., human immunodeficiency viruses and severe acute respiratory syndrome coronavirus 2). This could also explain how MCPyV may have been transmitted from apes to humans [32].

1.2 Seroprevalence

MCPyV prevalence study suggests that this virus is chronically shed from human skin representing part of the skin microbiota [33]. The initial exposure to MCPyV, based on the VP1 serology assay, supposedly occurs in early childhood. As reported in a study from Cameroon, significant titers against MCPyV were detected in newborns, although these titers decreased to undetectable levels by 16 months of age [34]. The maternal derived antibodies could represent the reason of the seropositivity in newborns. Moreover, these antibodies, effective in preventing primary infection, could explain why the seroprevalence is lower in children and higher in adults [34]. By 18 months of age, when the maternal antibodies were no longer detectable, children were susceptible to *de novo* infection and were able to mount an own antibody response. Beginning at 18 months of age, an increasing fraction of children became positive until approximately 80% tested positive at the age of 5 [34]. In a separate cohort from the same study, the correlation of seropositivity was observed between siblings of similar ages, suggesting that siblings likely were exposed to MCPyV at the same time and by each other [35]. These data suggest that transmission may occur via direct contact with the skin or saliva [34,35]. Several studies support the increasing risk with age for exposure and persistent infection by MCPyV [36-39]. A study conducted in Italy, with participants aged from 1 to 100 years old, showed how the seroprevalence for MCPyV rapidly increased with age: from 41.7% in children age from 1 to 4 years old, to 87.6% among

young adult (15-19 years old), remaining frequent in adulthood (79 to 96.2%) [40]. MCPyV seroprevalence studies performed in China (61% overall) and the Czech Republic (63%) yielded similar results with an increasing trend with age [41, 42]. Antibodies versus MCPyV LT and sT are detected in about 1% of healthy individuals and they can be present in patients with MCC [43]. Often MCC patients have higher titers of VP1 antibodies than normal healthy individuals [20].

1.3 Cell tropism: skin; replication in dermal fibroblasts

Because MCPyV was originally detected in MCC, a tumor believed to originate from Merkel cells (MCs), which are specialized skin cells, and is chronically shed from skin from healthy individuals, it was believed that the virus is *dermatotrophic*. It is now questioned that MCs are the target of MCPyV infection or productive replication because there are too few MC in the human skin to account for the millions of copies of MCPyV DNA detected on healthy skin [33]. Liu *et al.* speculated that the natural MCPyV host cells were one of the more abundant cell types in the human skin. They showed that human dermal fibroblasts support productive viral replication [44], and because MCs are situated in the basal layer of the epidermis near dermal fibroblasts, the authors hypothesized that MCPyV actively replicating in the dermal fibroblasts could accidently enter MCs and cause MCC [44]. Likely, MCs could represent a replication environment that supports viral integration and transformation [44]. It has also been demonstrated that MCPyV is capable of expressing LT and VP1 in fibroblast cell lines originating from lung tissue [44]. Hence, an active viral replication of MCPyV might be connected to all fibroblast tissues [44]. MCPyV DNA has been detected in cutaneous swabs [45] and it is possible that infected dermal fibroblasts might die and virions could be carried to the skin surface by the flow of differentiating keratinocytes [46]. This suggests that viral particles can be more widespread from the site of replication and release. This hypothesis is supported by the observation that MCPyV is frequently detected in eyebrow hair bulbs [47]. MCPyV can infect dermal fibroblasts near hair follicles and

it is possible that mature virions could be cleared to the surface of human skin through hair follicles and/or associated sebaceous and sweat glands [47].

1.4 MCPyV and MCC

MCC is a rare, neuroendocrine, cutaneous malignancy that was first described in 1972 by Toker as "trabecular carcinoma of the skin" [48]. The name was changed to MCC, since the tumor cells were similar to Merkel cells, present in particular around hair follicles and in the basal layer of the epidermis. Although MCC is a rare skin cancer, it is highly aggressive displaying a mortality rate of ~45% [49]. Consequently, MCC has a case-fatality rate higher than observed with melanoma [49]. Almost one third of the patients, at primary diagnosis, present loco regional metastases or lymph node metastases [49]. During the last 10 years, MCC incidence has increased significantly and is expected to increase further, since, the occurrence of this type of cancer, is related with aging (immune-senescence) and exposure to the sun [50]. An important alternative explanation for this finding is that before the large use of CK20 immunostaining, the pathology diagnosis was difficult and may at these ancient times require electronic microscopy, which was frequently not performed. Thus, true MCC were frequently misclassified [51,52]. The correlation between MCC and UV radiation is well documented [53]. Pigmentation of the skin seems to protect against MCC, as black, Asian and Hispanic individuals have considerably lower risk of MCC than white populations. Moreover, the occurrence of MCC is frequent in elderly patients on chronically sun-exposed skin, in individuals treated with UVA photo-chemotherapy and in patients with a history of other skin cancers associated with sun exposure. Melanoma is also linked with a three-fold greater risk of MCC [54]. A molecular UV signature, characterized by DNA mutations that are typically caused by UV damage, such as C to T transitions, has been demonstrated only in a subset of cases of VN-MCCs [55,56]. The association with UV exposure in VP-MCC could be related to other factors, such as UV-induced immunosuppression. In fact, immunodeficiency forms a risk factor in the development of MCC. MCC is more

frequent in patients with leukemia [57] or HIV infection [58] and in those who are immunosuppressed, as a result of organ transplantation or other causes [59]. The mortality is higher in immunosuppressed individuals than in immunocompetent patients [60]. These findings emphasize the crucial role of an efficient immune surveillance in the control of tumor growth and progression.

While ultraviolet radiation induced DNA damage is implicated in VN-tumors, the major causative factor of the MCC is considered MCPyV [61]. MCPyV was first identified in 2008, through whole-transcriptome sequencing [62], integrated into the genome of eight out of ten tested MCC cells. The Southern blot patterns of the primary tumor and a metastatic lymph node, isolated from the same patient, demonstrated an identical viral DNA integration at several different chromosomal sites. This important finding indicated that the viral integration was clonal and it was an early, if not initiating event, in VP- MCC oncogenesis process [62]. In addition, a C-terminal tLT form, lacking the OBD and helicase activity of LT required for viral DNA replication, was also observed [62]. Numerous studies have now confirmed that 80% of the examined tumors contain clonally integrated copies of the virus and express tLT [62-64]. MCPyV integration into the host genome occurs by accidental genome fragmentation during viral replication, in random site, without involvement of cellular tumor suppressor genes or oncogenes [56]. Viral integration involved mutations that result in the truncation of LT and a study by Schrama *and co-workers* suggests that truncating mutations occur before or during integration [65]. *In vitro* cell studies have demonstrated that expression of full-length LT in VP-MCC causes a specific DNA damage response, which is probably induced by *in situ* replication of the integrated viral DNA, which in turn is triggered by the binding of LT to the MCPyV ORI. Truncation of LT abolishes viral replication and seems to be necessary for MCC oncogenesis [5,66]. Tumor-derived tLT preserves the N-terminal J domain and LXCXE motif, whereas the DNA binding, helicase and cell growth-inhibitory domains are lost [66]. The tLT could potentiate a stable

integration of the MCPyV into the host genome [66]. All VP-MCC tested contain ≥1 viral genome copies/cell [65,67-70], whereas in *non*-MCC tumors that contain MCPyV, the viral load was at least 2-3 logs lower (reviewed in [61]).

1.5 Cell of origin of MCC

It was originally proposed that MCC derived from MCs because of similar immunophenotypes [71]. Both cells express cytokeratin 20 [72], synaptophysin [73], neural cell adhesion molecule/CD56 [74], and numerous endocrine markers [75]. However, it is more and more unlikely that MC are the cells of origin because several characteristics of MCC argue against MC as the progenitor cell of MCC. Epithelial, fibroblastic, lymphoid, and neural crest origin of MCC has been put forward (reviewed in [76-80]. Arguments in favor or contra these cell types as origin of MCC are summarized in **Table 1**.

Table 1. Supportive and contradictory arguments for the different hypotheses on cell of origin of MCC. The suspected cell of origin of VN-MCC and VP-MCC is indicated.

Cell of origin	Supporting cell of origin	Arguing against cell of origin
Merkel cell	1. Neuroendocrine granules 2. CK20 expression 3. Piezo 2 expression 4. Other neuroendocrine markers such as CD56, chromogranin A, synaptophysin, insulinoma-associated protein 1	1. Epidermal location 2. Postmitotic cells 3. Diffusely arranged skeleton 4. c-KIT, PAX-5, SCF, BCL2, CD24 are commonly expressed in MCC, but absent in MC 5. Neural cell adhesion molecule L1 (CD171) and neurofilament in MCC but not MC 6. Vasoactive intestinal peptide and metenkephalin expressed in MC but not MCC 7. Diffuse CK20 staining in MC; dot-like staining in MCC could not infect CK20-positive MC

		8. Not infected by MCPyV 9. Mouse models of VN- and VP-MCC[#] using MC-specific Cre drivers do not develop MCC
(Epi)dermal stem cell	1. Neuronal cell markers 2. CK14 expression 3. CK19 expression 4. SOX-2 expression 5. Mitotic potential 6. Other epidermal markers such as EMA, CK5/6, and BerEP4 7. VN-MCC harbor UV mutational signature characteristic of epidermal-derived cancers	1. Expression of B-cell markers 2. SOX-2 more widespread expression 3. Absence of MCPyV DNA in these cells 4. CK19 also found in MC
Pro/pre-B cell	1. B-cell specific lineage factors such as PAX-5, c-KIT, TdT, SCF, RAG1 2. Expression Ig (VP-MCC) 3. IgH and Ig◎ rearrangements (VP-MCC) 4. MCC regression with idelalisib treatment	1. Neurendocrine granules 2. Location of MCPyV transduction
Skin-derived precursors	1. Dermal location 2. Broad differentiation	1. Absence of MCPyV DNA in these cells
Dermal fibroblasts	1. Permissive for MCPyV 2. Mutational burden similar to VP-MCC 3. Mutational signature similar to VP-MCC 4. Only cell type that can be transformed by sT in vitro	1. Gene expression profile 2. Neuroendocrine differentiation 3. Expression of B-cell markers 4. Lack of MCPyV DNA in HDF adjacent to VP-MCC

Keratinocytes	1. Keratinocytes and MCs are derived from the same epidermal progenitor cell 2. Transgenic mice expressing LT or sT in keratinocytes can result in oncogenic effects 3. Mutational burden of VN-MCC is in line only with two other cancers, both keratinocyte-derived skin cancers 4. VN-MCC have mutations in *NOTCH1*, *HRAS* and *FAT1*, which are frequent in SCC[#]	

*TdT expression in 65% of all examined MCC and expression is significantly correlated with the presence of MCPyV.; PAX-5:90% of all examined MCC.

VP-MCC may also originate from different cell types than VN-tumors. Dermal fibroblasts were suggested since they are permissive for MCPyV infection [44], but also keratinocytes could be the cell of origin of VP-MCC because keratinocyte-specific expression of MCPyV oncoproteins resulted in oncogenic effects [81]. Other studies suggest that VN-MCC derive from epidermal keratinocytes, whereas VP-MCC derive from dermal fibroblasts [78,79]. A recent report supports the assumption that VP-MCC may derive from the epithelial lineage [82]. The authors sequenced a combined tumor of trichoblastoma (neoplasm of epithelial follicular germinative cells) and VP-MCC. Non-integrated viral DNA encoding full-length LT could be amplified from the trichoblastoma, while integrated virus (~20 copies/cell) was detected in the MCC. Remarkably, two different tLT may be expressed in this MCC tumor. Whole genome sequencing identified six somatic mutations common for both tumors. The trichoblastoma had expression of KRT17 and SOX9, and activation of GLI1 as observed by nuclear localization, markers that are shared with MC progenitors [83-85]. Therefore, the authors suggest that the trichoblatoma cell in which MCPyV integration occurred and led to the development of MCC could be an epithelial progenitor cell of the hair follicle or an already differentiated MC [82].

1.6. The oncogenic mechanisms of MCPyV T antigens

1.6.1 MCC cell growth depends on LT but not sT

Since the early proteins of other HPyVs possess oncogenic potentials in cell cultures and in animal models [86], the role of LT and sT in tumor growth was examined. Knock down of sT and LT (i.e. truncated LT and 57kT which cannot be distinguished in most VP-MCC cell lines) reduced MCC cell proliferation in culture, but also in xenograft mice [87-89]. Specific knockdown of only LT was sufficient to generate growth inhibition. Rescue experiments, i.e. expression of T antigens in cells where their endogenous expression was knocked down showed that wild-type sT plus LT could rescue cell growth. The growth promoting property of LT involves binding to RB because mutations in the DnaJ domain, the RB domain, or S220A abrogates LT's ability to promote cell growth [87,90,91].

Ectopic expression of the tLT variant MKL-1 in MCC13 promoted cell cycle progression [89]. However, RNA interference studies showed that sT is dispensable for growth and survival of VP-MCC cell lines [89]. Interestingly, knockdown of the T antigens in the VP-MCC LoKe cell line did not results in any growth inhibition. The authors speculate that additional aberrations enable cell growth even in the absence of T antigens and therefore, in some VN-MCC cases a viral hit-and-run mechanism was possible where MCPyV initiates tumor formation, then disappears, but additional mutations drive tumor progression and maintenance [92]. Studies in mouse and human fibroblasts demonstrated that expression of a tumor-derived tLT has stronger growth promoting activities than wild-type LT and 57kT [93]. Expression of full-length did not induce anchorage-independent growth, whereas tLT proteins induced aggregates in soft agar that did not grow into full colonies, suggesting that tLT has increased cell proliferative capacity compared with the wild-type LT. Expression of the C-terminal 100 amino acids residues inhibited the cell growth of fibroblasts and of the VP-MCC MKL-1 cell line [93]. The mechanism by which this region inhibits cellular growth is not known, but is likely to be independent of p53 since neither full-length LT nor 57 kT-are able to bind p53. The

C-terminal domain may interact with yet an unidentified cellular protein involved in growth regulation. Putative candidates are the cell cycle checkpoint kinase ATM, casein kinase 2 and phosphatidylinositol-5-phosphate 4-kinase type 2◎, which are all involved in proliferation and were found to interact with MCPyV LT, but the biological importance of these interactions were not examined, nor was the region of LT required for interaction identified (reviewed in [21]). CRISPR/Cas9 targeting of LT/57kT impaired MS-1 and WaGa cell proliferation, decreased G1/S cell cycle progression and increased apoptosis. Additional targeting of sT did not enhance the effect in LT/57kT mutated cells [94].

1.6.2 Oncogenic properties of LT

Cell culture studies revealed that neither full-length nor tumor-derived tLT was able to trigger cellular transformation [95], but LT is required for growth of VP-MCC cells [90,96]. The C-terminal domain of LT causes DNA damage and stimulates host DNA damage response, leading to p53 activation and inhibition of cellular proliferation. Phosphorylation of the C-terminus by ATM kinase induces apoptosis and inhibits proliferation [14,66]. Thus, the C-terminus of LT contains anti-tumorigenic properties and may explain why this region is deleted in VP-MCC. To further elucidate the role of MCPyV LT in MCC tumorigenesis, cellular proteins that interact with LT were identified using different methods [21,97]. However, the biological relevance of these interactions and possible implications for MCPyV-induced cancer have not always been studied.

LT and p53

LT expressed in VP-MCC is truncated in its C-terminal part, which encompasses the p53-binding domain in LT of other HPyVs. As expected, tLT did not interact with p53, but surprisingly neither did full-length LT [93]. In another study, Borchert *and co-workers* showed that an antibody against p53 could immuno-precipitate full-length, but not tLT [98]. However, LT did not bind p53 directly and LT, but not tLT

inhibited p53-mediated transcription. They suggested that full-length LT interacts with a bridging protein that serves as a co-activator in p53-driven transcription. Alternatively, another protein may change the conformation of LT allowing it to bind p53 as has been shown for human papillomavirus E6 protein. E6 forms a complex with E6AP and p53, but neither E6 nor E6AP are separately able to recruit p53. However, E6AP renders the conformation of E6 competent for interaction with p53 [99]. Park *et al*. reported that expression of tLT in IMR90 lung fibroblasts significantly stimulated transcript levels of p53-responsive genes and increased both total protein and Ser-15 phosphorylation levels of p53 [100]. They showed that the interaction between LT and RB1 lead to increased levels of ARF and activation of p53. ARF is an inhibitor of the E3 ubiquitin protein ligase MDM2, which degrades p53 [101]. Hence, LT can through RB-ARF-MDM2 axis stabilize p53.

LT and Retinoblastoma (RB) family

Both full length and tLT interact with RB1, although with different strength [93,98,100]. This suggests that LT may usurp RB1, thereby relieving repression of E2F-mediated transcription and induce cell cycle progression into S phase. MCPyV LT did not interact with the p107 and p130 retinoblastoma family members, nor did it interfere with p107-induced and p130-induced cell cycle arrest and repression of E2F responsive genes [93,95,98]. The weaker *in vitro* oncogenic potentials of MCPyV LT compared to LT of other PyVs may be attributed to its weaker impact on the tumor suppressors p53 and RB.

LT and HSC70

LT interacts with HSC70 via the DnaJ domain and stimulates viral replication [12]. Like other PyVs, it is presumed that MCPyV LT disrupts Rb-E2F family complexes through the action of its DnaJ domain and ATPase activity of Hsc70 [102,103]. The biological significance of the DnaJ domain in sT is unknown as mutations in DnaJ of

sT did not interfere with its effect on viral replication or *in vitro* transformation activity [12,95].

LT and VPS39 subunit of HOPS complex/Vam6p

Human Vam6p, a cytoplasmic protein involved in lysosomal processing and clustering, interacts with MCPyV full-length LT as well as MCC-derived tLT [18]. LT and tLT that retains its nuclear localization signal translocate hVam6p to the nucleus and sequester it from involvement in lysosomal trafficking. The physiological consequences of LT:Vam6p interaction are not known, but it might play a role in MCPyV replication rather than tumorigenesis, because VP-MCC have been described that express tLT without a nuclear localization signal [62,104,105].

LT and ATOH1

Sox2 (sex-determining region Y-box 2) and Atoh1 (atonal homolog 1) are critical transcription factors for MC development in mice [106]. Harold *and colleagues* found that knockdown of all T antigen isoforms in VP-MCC cell lines co-cultured with human keratinocytes promotes a neuronal phenotype in the MCC cells and resulted in reduced expression of ATOH1 and SOX2 [107]. The tLT 339 variant stimulated ATOH1 and SOX2 expression levels, but neither a LT399 retinoblastoma binding deficient mutant nor sT increased expression of ATOH1 and SOX2. Activation of the SOX2-ATOH2 pathway by LT in a retinoblastoma-dependent manner is important for both the manifestation of a Merkel cell phenotype and tumorigenesis. Transcriptional activation by ATOH1 requires E-boxes (5-CANNTG-3') and E47 binding site [108], both of which are present in the miR-375 promoter. Indeed, ATOH1 stimulated expression of miR-375 and ectopic expression of tLTs stimulated the activity of a minimal promoter containing three E-box and induced *ATOH1* mRNA and miR-375 in fibroblast MRC-5 cells [109]. Moreover, high transcript levels of *LT* and *ATOH* were detected in the VP-MCC WaGa cells. sT, however, was unable to enhance *ATOH1* mRNA and miRNA-375 levels. The neuroendocrine features of

MCC may therefore be linked to MCPyV-induced expression of ATOH1. Whether LT-induced expression of miR-375 is exclusively mediated by ATOH1, or also by an ATOH1-independent mechanism remains to be elucidated. As both *ATOH1* and miR-375 promoters were hypomethylated, LT may stimulate demethylation of these promoters. Finally, strong expression of ATOH1 and miR-375 was also observed in classical VN-MCC cells, indicating a virus-independent mechanism in their expression [109].

LT and ubiquitin-specific protease 7 (Usp7)

All MCPyV T-antigens interact with Usp7, a cellular deubiquitination enzyme [110]. The binding with LT, tLT and 57kT is direct, whereas sT probably interacts indirectly. Binding of Usp7 required the tumor necrosis factor receptor-associated domain of Usp7 and did not alter the ubiquitination levels of the T antigens, but stimulated the binding affinity of LT to the ORI, thereby restricting viral DNA replication. Usp7-mediated restriction of MCPyV replication could promote viral persistence [110]. Whether Usp7-T antigens interaction contributes to MCC tumorigenesis remains elusive. However, interference with other functions of Usp7 such as DNA damage response, epigenetic regulation, and immune response may also play a role in the development of virus-induced MCC [111].

LT and other interacting proteins

Other interaction partners of MCPyV LT are summarized in **Table 2**. The interaction in VP-MCC has not been validated and the biological consequences of these interactions have not been investigated.

Table 2. MCPyV LT and sT interaction partners and their role in the life cycle of MCPyV. See text for details.

T antigen	Protein	Functional class	Biological role	Reference
sT	abhydrolyse domain containing 12 (ABHD12)	Metabolism	unknown	[112]
sT	ankyrin repeat domain 13A (ANKRD13Aa)	Protein stability	unknown	[112]
sT	ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2	Metabolism	unknown	[112]
LT	ATP binding cassette subfamily A Member 13 (ABCA13)	Signaling	unknown	[112]
sT	ATP binding cassette subfamily D member 3 (ABCD3)	Signaling	unknown	[112]
LT	ATP binding cassette subfamily D member 13 (ABCD13)	Signaling	unknown	[112]
sT	aryl hydrocarbon receptor interacting protein (AIP)	Transcription	unknown	[112]
LT	adaptor related protein complex 2 subunit A and M (AP2A1 and M1)	intracellular transport	unknown	[112]
sT	ADAM metallopeptidase domain 9 (ADAM9)	cytoskeleton/extracellular matrix	unknown	[112]
LT	ataxia telangiectasia mutated (ATM kinase)	DNA replication and repair	LT phosphorylation	[14]
LT, sT	BCL2 associated anthanogene 2, 3 and 5 (BAG2, 3 and 5)	protein stability/apoptosis	unknown	[112]
LT	bromodomain protein 4 (Brd4)	cell cycle/DNA replication	viral genome replication	[113,114]
sT	cadherin 1 (CDH1)	cytoskeleton/extracellular matrix	unknown	[112]
LT	casein kinase 2 beta	Signaling	unknown	[112]
sT	cathepsin B (CTSB)	protein stability/modification	unknown	[112]
LT	caveolae associated protein 2 (CAVIN2)	intracellular transport	unknown	[112]
sT	CCHC-type Zinc finger nucleic acid binding protein (CNBP)	Transcription	unknown	[112]

sT	cell surface glycoprotein 44 (CD44)	cell-cell interaction, cell adhesion, migration	unknown	[112]
sT	cell division cycle 20 (CDC20)	cell cycle	sT-mediated phosphorylation of 4E-BP1	[112,115,116]
sT	coatomer protein complex subunit ⊚2	intracellular transport	unknown	[112]
sT	2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNP)	nucleotide metabolism	unknown	[112]
LT	DEAD-box helicase (DDX24)	post-transcription/translation	unknown	[112]
sT	heat shock protein 40 members A1 and B4 (DnaJA1 and B4)	Chaperone	unknown	[112]
LT, sT	heat shock protein 40 member C7 (DnaJC7)	Chaperone	unknown	[112]
LT	transcription factors E2F3 and 4 (E2F3 and 4)	transcription	unknown	[112]
sT	EGF containing fibulin extracellular matrix protein 2 (EFEM2)	cytoskeleton/extracellular matrix	unknown	[112]
sT	eukaryotic translation initiation factor 4E binding protein 1 (eIF-4EBP1)	Translation	dysregulated cap-dependent translation which promotes tumorigenesis	[95,116]
LT, sT	emerin (EMD)	Cytoskeleton	unknown	[112]
LT	family with sequence similarity 71 member E2 (FAM71E2)	Unknown	unknown	[112]
sT	F-box and WD repeat domain containing 7 (Fbxw7)	protein stability	tumorigenic properties of the virus (stabilization of LT and cellular proteins)	[117,118]
LT	general transcription factor IIIC subunit 1 (GTF3C1)	transcription	unknown	[112]
LT	high density lipoprotein binding protein (HDLBP)	Metabolism	unknown	[112]
LT, sT	heat shock protein 70 (HSPA1 and A4)	Chaperone	cell cycle progression	[112]
sT	insulin like growth factor 2 receptor (IGF2R)	Signaling	unknown	[112]
LT, sT	inhibitor of nuclear factor kappa-B kinase-interacting protein (I⊚BIP)	Signaling	unknown	[112]

LT	karyopherin subunit 2, 3 and 4 (KPNA2, 3 and 4)	intracellular transport	unknown	[112]
sT	lysyl oxidase (LOX)	Metabolism	unknown	[112]
LT	microtubulin-associated protein 4 (MAP4)	Cytoskeleton	unknown	[112]
sT	membrane bound O-acetyltransferase domain containing 7	metabolism/plasma membrane lipid organization	unknown	[112]
LT	mediator complex subunit 14 (MED14)	transcription	unknown	[112]
sT	matrix metalloproteinase 14	extracellular matrix	unknown	[112]
sT	myelin protein zero like 1 (MPZL1)	Signaling	unknown	[112]
sT	mitochondrial carrier 2 (MTCH2)	Metabolism	unknown	[112]
sT	myoferlin (MYOF)	membrane morphology	unknown	[112]
sT	NF-kappa-B esssential modulator (NEMO(=IKBKG)	Signaling	inhibition NFkB signaling; immune evasion	[119]
sT	Notch 2 receptor (NOTCH2)	Signaling	unknown	[112]
sT	nuclear receptor binding SET domain protein1 (NSD1)	Transcription	unknown	[112]
LT	prolyl 4-hydroxylase subunit alpha 3 (P4HA3)	Metabolism	unknown	[112]
sT	prolyl 4-hydroxylase subunit P4HB)	Metabolism	unknown	[112]
sT	platelet-derived growth factor receptor subunit (PDGFR)	Signaling	unknown	[112]
LT	PGAM family member 5, mitochondrial Ser/Thr protein phosphatase (PGAM5)	Signaling	unknown	[112]
sT	progesterone receptor membrane component 2 (PGRMC2)	Signaling	unknown	[112]
LT	phosphatidylinositol-5-phosphate 4-kinase type 2 beta (PIP4K2)	Signaling	unknown	[112]
LT	protein phosphatase 2 scaffold subunit PP2AR1)	Signaling	unknown	[112]
sT	protein phosphatase 2 catalytic subunit (PPP2CA and CB)	Signaling	mutation in PP2A binding site had no effect on the known activities of sT	[17,119,120]

sT	PRA1 domain family member 2 (PRAF2)	intracellular transport	unknown	[112]
sT	protein phosphatase 2 regulatory subunit Aα and Aβ (PP2R1A and B)	Signaling	unknown	[121]
sT	protein phosphatase regulatory subunit 1 (PP4R1)	Signaling	microtubule destabilization and cell motility (metastasis?); inhibition NFκB signaling (immune evasion?)	[17,119,122,123]
sT	protein phosphatase Mg^{2+}/Mn^{2+} dependent 1A, 1B and 1G (PPM1A, B and G)	Signaling	unknown	[112]
sT	proteasome 26S ATPase 2,3 and 4 (PSMC2, 3 and 4)	protein stability	unknown	[112]
LT	caveolae associated protein 1 (PTRF)	transcription	unknown	[112]
sT	pituitary tumor-transforming gene 1 protein-interacting protein (PTTPG1P)	intracellular transport	unknown	[112]
sT	Rab18 (RAB18)	Signaling	unknown	[112]
LT	Retinoblastoma protein 1 (RB1)	cell cycle	cell cycle progression	[93,98,100]
sT	ribonuclease/angiogenin inhibitor 1 (RNH1)	transcription/translation	unknown	[112]
sT	ribosomal protein L21	Translation	unknown	[112]
sT	ribosomal protein S27 like	Translation	unknown	[112]
LT	recitulon 4 (RTN4)	intracellular transport	unknown	[112]
LT	sphingosine-1-phosphate lyase 1 (SGPL1)	Metabolism	unknown	[112]
sT	secreted protein acidic and cysteine rich (SPARC)	extracellular matrix	unknown	[112]
sT	sulfide quinone oxidoreductase (SQRDL)	Metabolism	unknown	[112]
LT	signal recognition particle 14 (SRP14)	intracellular transport	unknown	[112]
LT, sT	signal recognition particle receptor subunit b (SRPRB)	intracellular transport	unknown	[112]
sT	ser/thr kinase 38 (STK38)	Signaling	unknown	[112]
LT, sT	STIP1 homology and U-box containing protein 1 (STUB1)	protein stability	unknown	[112]
sT	surfeit 4 (SURF4)	intracellular transport	unknown	[112]

LT	Ubiquitin-specific protease (USP7)	protein stability	inhibition viral DNA replication	[110]
LT	transcription elongation factor B subunit 1 (TCEB1)	Transcription	unknown	[112]
LT	transcription factor DP1 (TFDP1)	transcription	unknown	[112]
sT	translocase of inner mitochondrial membrane 8A (TIMM8A)	intracellular transport	unknown	[112]
sT	transmembrane protein 165 (TMEM165)	protein glycosylation	unknown	[112]
sT	thioredoxin related transmembrane protein 3 (TMX3)	protein folding	unknown	[112]
sT	toll interacting protein (TOLLIP)	Signaling	unknown	[112]
LT	tripartite motif containing 38 (TRIM38)	protein stability	unknown	[112]
LT	testis-specific Y-encoded-like protein 1 (TSPYL1)	Transcription	unknown	[112]
sT	tubulin α1 (TUBA1B)	protein folding and gap junctions	unknown	[17]
sT	tubulin $\beta 2\alpha$ (TUBB2A)	mitosis and intracellular transport	unknown	[17]
LT, sT	upregulated during skeletal muscle growth 5 (USMG5)	nucleotide synthesis	unknown	[112]
LT	VPS39 subunit Of HOPS complex (Vam6p)	intracellular transport	role in DNA replication (?)	[18,112]
LT	VAMP associated proteins A and B (VAPA and VAPB)	intracellular transport	unknown	[112]
sT	vitamin K epoxide reductase complex subunit 1 (VKORC1)	Metabolism	unknown	[112]
LT	vacuolar protein sorting-associated protein 11 homolog (VSP11)	intracellular transport	unknown	[112]

LT and sT induce microRNAs that target mRNAs for proteins involved in autophagy

Autophagy plays an important role in cancer and in immune evasion [124-126]. Silencing LT or LT+sT in VP-MCC cell lines reduced the expression of miR-30a-3p, miR-30a-5p and miRNA-375, while ectopic expression of tLT or sT in VN-MCC cells

increased the levels of these miRNAs. Induced expression of miR-30a-3p, miR-30a-5p and miRNA-375 required the DnaJ domain [127]. Target mRNA of these miRNAs encode the autophagy proteins ATG7, SQSTM1/p62 and BECN1. The authors showed that sT and tLT, but not wild-type LT suppressed autophagy processes in MCC cells and protein levels of ATG7 and SQSTM1/p62 were lower in VP-MCC compared with VN-MCC. Hence, T antigens-mediated suppression of autophagy might protect cancer cells from cell death and contribute to tumorigenesis [127].

1.6.3 The role of 57kT and ALTO in VP-MCC

Whether 57 kT and ALTO are implication in MCPyV-induced tumorigenesis remains to be established. The 57kT protein retains the RB binding domain and the CR1 and DnaJ binding motifs. Immortalized human fibroblasts Bj-hTERT expressing 57kT grew slower than control cells and when LT cDNA was stably expressed in mouse and human fibroblasts, the 57kT form was preferentially expressed [128]. Expression of 57 kT has never been detected in VP-MCC [68,69], but due to truncation in the LT gene, LT and 57kT cannot be distinguished in most MCC using the antibodies currently available. The role of 57kT in MCC remains unsolved. Deletion of ALTO did not abrogate viral replication and is dispensable for MCPyV-driven tumor cell proliferation [3,90], but the function of this protein remains elusive.

1.6.4 The oncogenic properties of sT

MCPyV sT is sufficient to fully transform Rat-1 and NIH3T3 mouse fibroblasts [89,95,129]. Knockdown of sT expression in VP-MCC cell lines causes cells to stop proliferating, but did not result in cell death. Co-expression of full-length or tLT did not enhance sT-induced colony formation compared with expression of sT alone [95].

sT and transgenic mice

Considering the non-transforming potentials of LT in cell culture and that sT can induce transformation, sT, but not LT transgenic mice models have been generated. Verhaegen *et al.* generated a transgenic mouse model in which sT expression was regulated by the epidermis-specific keratin-5 promoter [117]. Analysis of embryos revealed that sT promotes neoplastic transformation in epithelia in a PP2A-independent, but LSD-dependent manner. Adult animals developed lesions strongly resembling squamous cell carcinoma *in situ*. However, expression of sT alone does not appear to be sufficient to drive epidermal cells in MCC in a mouse model. The same group generated K5-tLT, K5-sT+tLT, K5-st+Atoh1, K5-tLT+Atoh1, K5-sT+tLT+Atoh1, and K5-Atoh1 transgenic mice [130]. The tLT embryo had no apparent phenotype, co-expression of sT+Atoh1 resulted in MCC-like tumors, and co-expression of tLT did not noticeably altered the phenotype of sT or sT+Atoh1 mice. These studies indicate that Atoh1-induced differentiation of epidermal cells into neuroendocrine lineage together with sT as the viral oncogenic driver can result in MCC development [130]. Transgenic mice co-expressing sT and tLT under control of the keratinocyte-specific K14 promoter developed hyperplasia, hyperkeratosis and acanthosis, and some mice develop papillomas, but not MCC [79]. Shuda *and c-workers* developed a sT-p53-Atoh1 transgenic mice which allowed sT expression in MC cells [131]. Although these mice have increased embryonic MC precursor proliferation, they did not develop MCC. Taken together, *in vitro* and animal studies and the detection of sT in the absence of LT in some VP-MCC indicate that sT may be more involved in the oncogenic process, whereas LT is required to maintain the tumor cell growth [89,95]. However, studies in the genuine cells of origin of VP-MCC are required to determine the requirements of sT and LT in cell growth and oncogenesis.

sT and eukaryotic translation initiation factor 4E binding protein (4E-BP1)

Transcription initiation factor 4E-BP1, a downstream target of the Akt-mTOR pathway, binds in its un-phosphorylated or hypo-phosphorylated form eukaryotic initiation factor 4E (eIF4E), thereby preventing assembly of eIF4F onto capped mRNA and inhibiting translation [132]. sT interacts with 4E-BP1 and expression of sT, but not LT promoted 4E-BP1 phosphorylation [95,116]. sT-induced phosphorylation of 4E-BP1 is accomplished by sT interacting with Cdc20 and possibly Cdc20 homolog 1 (Cdh1), which activates the CDK1/cyclin B1 complex and CDK1 and phosphorylate 4E-BP1 [115,116]. 4E-BP1 hyper-phosphorylation was required for sT-induced transformation of rodent cells [95,116]. The importance of sT-mediated 4E-BP1 phosphorylation in MCPyV-induced MCC is not completely understood, but sT-induced hyper-phosphorylation of 4E-BP1 can dysregulate cap-dependent translation, an event that has been shown to promote tumorigenesis [133].

sT and E3 ubiquitin ligases

Binding of sT to E3 ubiquitin ligase complex SCF^{Fbw7} led to inactivation of the enzymatic activity and stabilization of LT, which is a substrate of SCF^{Fbw7} [118]. Binding occurs through LSD and loss of net positive charge in the LSD abrogated sT:SCF^{Fbw7} interaction [129]. sT-induced stabilization of LT stimulates viral replication and transformation of rodent fibroblasts cell cultures by sT is SCF^{Fbw7}-dependent [129,134], and increased protein levels of SCF^{Fbw7} substrates Mcl-1, c-Jun, mTOR and cyclin E in sT transgenic mice [117]. sT also interacts with the E3 ubiquitin ligases Cdc20-anaphase promoting complex [17] and β-TrCP [135] and this stimulated genome instability [118]. Inactivation of E3 ubiquitin ligases by sT may be therefore be an important contributor in MCPyV-induced transformation and tumorigenesis. However, Dye and colleagues failed to detect interaction between sT and SCF^{Fbw7} and sT and β-TrCP and no increased c-Myc levels were observed when

sT was overexpressed. They also demonstrated that sT-mediated stabilization of LT did not require SCF^{Fbw7} [136]. The reason for the discrepancies between the different students is presently unknown. sT can form a complex with the E3 ubiquitin ligase STIP1 homology and U-box containing protein 1 (STUB1) [112]. This E3 ubiquitin ligase plays also a role in innate and adaptive immunity [137], but the biological implications of sT:STUB1 interaction in MCPyV replication and MCC remain to be determined.

sT and N-myc downstream regulated gene-1 (NDRG1)

Stable expressing the entire MCPyV early region in human immortalized keratinocytes resulted in >1.5-fold up- or down-regulated of 325 genes [138]. Of these, 73 had decreased expression and the majority encodes proteins involved in cell senescence, DNA repair, signal transduction, and cell cycle regulation, including HIST1H1C. Upregulation of HIST1H1C was also confirmed in VP- and VN-MCC cell lines, MCC tumors, and in sT expressing human fibroblasts expressing compared with normal fibroblasts [139-141]. Of the upregulated genes, many encode proteins implicated in cell cycle regulation and signaling pathways, including CDK4, cyclins D2 and D3, CDC25, FOXQ1, DUSP10, and CTSH. One gene that was specifically down-regulated by MCPyV, but and not by other HPyVs and SV40 was the *N-myc downstream regulated gene-1* (*NDRG1*). NDRG1 is a known tumor suppressor and metastasis suppressor [142]. Knock-down of sT+LT in MKL-1, MKL-2, MS-1 and CVG-1 increased NDRG1 levels in all four cell lines, and decreased cyclin D1 and CDK2 levels in MKL-2, MS-1, and CVG-1 cells. Overexpression of NDRG1 in MKL-2 reduced cyclin D1 and CDK2 levels, but not in MKL-1 cells. The different status of transformation of may explain the difference between MKL-1 and the other VP-MCC cell lines. Depletion of sT alone or sT+LT resulted in comparable increase in *NDRG1* mRNA levels, suggesting that sT is sufficient. Overexpression of NDRG1 in keratinocytes stably expressing MCPyV early region or in MKL-1 and MKL-2 cells inhibits cellular proliferation and migration. Taken, together these

observations indicate that MCPyV-mediated repression of NDRG1 participates in MCC tumorigenesis and that sT may be the main contributor. The expression levels of NDRG1 have not yet been examined in VN- and VP-MCC. In a study in 91 MCC tumors (30 VN and 61 VP), cyclin D1 expression was only detected in two tumors, both of which were MCPyV negative [143].

sT and p53

LT indirectly activates p53 (see above) and sT can stabilize LT, yet co-expression of LT and sT reduced p53 activation [134]. MCPyV sT can inhibit p53 activity indirectly by binding to and activating the transcription factor MYCL and the histone acetylase complex EP400 [98]. The MYCL:EP400 complex controls transcription of *MDM2* and *CSNK1A1* genes. The latter encodes casein kinase 1α which activates MDM4, an inhibitor of p53 [144]. The activation of p53 by LT may exert anti-tumorigenic effect, while sT-mediated inhibition of p53 favors pro-tumorigenesis. The relative concentrations of LT and sT, but also the strength of impact of LT and sT on p53 will determine the outcome. VP-MCC cells have been shown to express high levels of MDM4 [100]. Accordingly, p53 levels were found to be lower in VP-MCC cell lines compared to VN-MCC cell lines [100,145,146]. Examination of MCC revealed that mutations in *TP53* gene are almost exclusively detected in VN-MCC, but only 7% of VP-MCC expressed detectable p53 levels and an inverse correlation between p53 expression and viral DNA copy number was observed [143,147]. One study reported that p53 levels were variable between patients, with no obvious differences between VN- and VP-tumors [148]. The expression levels of MDM4 in VN- and VP-MCC biopsies have not yet been examined. Another consequence of the interaction of sT with MYCL:EP400 complex that may be involved in tumorigenesis was recently published. This complex stimulates the expression of components of the lysine-specific histone demethylase 1 (LSD1) complex that acts as a transcriptional repressor [100]. Treatment of VP-MCC cell lines with LSD1 inhibitors completely blocked colony formation in soft agar, and LSD1 inhibitors reduced the growth of

MCC *in vitro* and in xenograft models using VP-MCC cells. Hence, sT-mediated activation of the LSD1 complex seems to play a pivotal role in VP-MCC, and LSD1 inhibitor could be used to treat VP-MCC patients.

sT and protein phosphatases

Because aberrant or loss of enzymatic activity of protein phosphatases (PPs) can lead to transformation and their role in cancers, PPs are considered tumor suppressors and are targeted by several tumor viruses [149-153]. MCPyV sT interacts with PP1A, 1B and 1G [17,112]. The biological consequences of sT:PP1 interaction have not been determined, but RB is a PP1 substrate. Inhibition of PP1 by sT may therefore results in hyperphosphorylation of RB, release of repression of E2F target genes, and drive to enter the S-phase [154].

PP2A exists as a heterotrimer composed of a structural subunit A, a regulatory subunit B, and a catalytic C subunit [155]. MCPyV sT binds the structural subunit Aβ and Aα, and the catalytic subunits Cα and Cβ. This binding reduced the catalytic activity of the enzyme [17,119-121]. sT's binding to PP2A excluded the regulatory subunit B56α, but not other B subunits [17]. The biological implications of the sT: PP2A interaction are not known because mutations in sT that prevented PP2A binding had no effect on sT's transforming activity [95], nor did it impede sT-induced skin hyperplasia in transgenic mice [117].

MCPyV sT was reported to interact with PP4 [17,119,122,123], and this interaction promotes microtubule destabilization and stimulates cell motility and filopodium formation [122,123]. The sT: PP4 association also interferes with the NFκB pathway. The transcription factor NFκB is retained in an inactive state in the cytoplasm through interaction with inhibitor of κB (IκB). Activation of the NFκB pathway occurs after phosphorylation of IKB by IκB kinase (IKK) and subsequent degradation of IKB. IKK is a trimeric complex that consists of IKKα, IKKβ, and IKKγ or NFκB essential modulator (NEMO). Release of NFkB allows nuclear translocation where it affects transcription of NFκB-responsive genes [156]. NFκB target genes

encode proteins involved in inflammation, immune responses, including antiviral response [155,158]. Griffith *and colleagues* demonstrated that sT associates with a PP4R1-PP4C complex, which stimulates the interaction between NEMO and the protein phosphatase PP4C-PP4R1 complex. Consequently, NEMO-mediated recruitment of PP4C to the IKK complex reduces IKK phosphorylation, with subsequent inhibition of IκB and failure to release, activate (phosphorylate), and translocate NFκB to the nucleus [119]. Thus, MCPyV may affect inflammatory and immune responses by interfering with the NF◎B pathway. However, the importance of the sT:NF◎B interaction in tumorigenesis is questioned because a significantly higher expression of pSer-536 RelA/p65 subunit of NFκB was observed in VP- (n = 24) compared to VN-MCC (n = 17). The phosphorylated p65 form was exclusively detected in the nucleus [159].

sT and sheddases

MCPyV sT stimulates expression of the sheddases ADAM10 and 17, proteins involved in cell signaling, inflammation, and tumor formation and progression [160]. The exact mechanism by which sT enhances ADAM 10 and ADAM17 expression is not known, but sT increases expression of the transcription factors ACAD8, PPARG, and ITGB3BP that activate the ADAM10 promoter [141]. ADAM 10 and 17 protein levels are higher VP-MCC tumors compared to VN-MCC, suggesting that sT-induced sheddase expression may contribute to MCC progression [160].

sT and metabolism

Ectopic expression of sT in normal human fibroblasts IMR90 resulted in significantly perturbed metabolism with elevated aerobic glycosylation and upregulation of transcription of metabolite transport genes [141]. Proteins whose transcripts were significantly upregulated included monocarboxylate lactate transporter 1 (MCT1), glucose transporter GLUT1, and GLUT3. Inhibition of MCT1 activity suppressed the

growth of VP-MCC cell lines and impaired MCPyV-dependent transformation of IMR90 cells. Berrios *et al.* showed that MYCL cooperates with the tumor derived MCPyV early region (expressing sT and tLT) to induce expression of MCT1 and knockdown of the p65 subunit of NF◎B reduced sT, as well as sT+MYCL stimulated MCT1 expression. Taken together, these data suggest that sT-mediated changes in the metabolic state are implicated in virus-induced MCC tumorigenesis. MCT1 expression levels in VN- and VP-MCCs have not been examined, but inhibitors of MCT1 could be considered to treat VP-MCC [141].

sT and other interaction partners

Other cellular proteins reported to interact with MCPyV sT are shown in **Table 2**. The interaction in genuine host cells for MCPyV and in VP-MCC has not been confirmed, nor has the physiological relevance of these interactions been explored.

1.6.5 Effect of MCPyV on signaling pathways in MCC

The phosphatidyl-3-kinase/AKT/mammalian target of the rapamycin (PI3K/AKT/mTOR) pathway

The PI3K/AKT/mTOR pathway, which plays pivotal roles in cell growth, motility, survival, metabolism, and angiogenesis is often the target of viral infections [161,162]. Strong staining with phosphoT308 AKT antibodies was observed in most of the MCC samples examined, but there was no significant correlation between phosphoAKT and MCPyV status [163,164]. Another study reported AKT phosphorylation in 4 VN-MCC cell lines, but not in VP-MCC cell lines [165]. However, three of the tested VN-MCC cell lines (MCC13, MCC26 and UIOS) are non-classical MCC cell lines High expression levels of PI3K and PI3Kδ were observed in respectively 20% and 52% of archival MCC specimens (n=50) [166]. The viral status in the MCC samples was not described, but PI3K δ transcript levels were detected in 2 VN and 2 VP-MCC cell lines, while one of the VP-MCC cell lines (MKL-1) had no detectable PI3K δ mRNA levels. This suggests that the expression levels of

PI3K do not depend on the presence of MCPyV, which is underscored by the finding that silencing of LT and sT in four MCPyV positive MCC cell lines had no effect on AKT phosphorylation [163]. Taken together, the results indicate that activation of AKT in MCC is not caused by MCPyV. A well–known substrate of the PI3K/AKT/mTORC1 pathway is 4E-BP1 and its interaction with sT was discussed earlier.

Protein kinase C pathway

Protein kinase C (PKC) is family of serine/threonine kinases that comprises PKCα, βI, βII, γ, δ, ε, η, θ, ζ and ι [167]. Because PKCε plays critical roles in cancer [168], its activation (i.e. phosphorylation of Ser729) was examined in 8 VP-MCC and three VN-MCC specimens [169]. Seven of the VP-MCCs were positive for phospho-PKCε, whereas only one of the three VN-MCC samples expressed phospho-PKCε. These results suggest a correlation between PKCε activation and MCPyV positivity in MCC. However, relative few samples were examined and the involvement of MCPyV in PKCε activation remains to be proven.

Notch pathway

There are four human Notch receptors (NOTCH1–4) and their ligands include Jagged 1 and 2, and Delta-like proteins [170]. Relative expression levels of NOTCH1, NOTCH2, NOTCH3, and Jagged 1 were compared in 19 VN- and 19 VP-MCC tumors [171]. NOTCH3 expression was higher in VP-MCC compared to VN-MCC, while the opposite was found for Jagged 1. Patients with higher NOTCH3 expression had better overall survival, whereas expression of NOTCH1 and NOTCH2 was not associated with MCPyV status or prognosis. Whether MCPyV proteins are implicated in the upregulation of NOTCH3 and downregulation of Jagged 1 remains to be investigated. MCPyV sT can bind NOTCH2, but the functional implication of this interaction is not known [112]. sT may also activate the NOTCH pathway through stimulating the expression of ADAM10 [160].

Hedgehog signaling pathway

Patched 1 (PTCH1) is the receptor for the hedgehog ligand of which 3 are found in humans: sonic (SHH), Indian (IHH), and desert (DHH) hedgehog [172]. Expression of SHH and IHH was monitored in 29 VP-MCCs and 21 VN MCCs. A significant higher expression of SHH and IHH was observed in the VP-MCCs than in VN-MCCs [173].

Apoptotic pathway

Expression of pro-survival proteins Bcl-2, $BclX_L$, Bcl-w, Mcl-1 and A1 has been investigated in both VN- and VP-MCC. High expression of these anti-apoptotic proteins was measured in most MCC and no correlation was found with the viral status of the tumor [145,174-178]. Despite high Bcl-2 levels in most tumors, a phase II clinical trial with Bcl-2 antisense RNA G3139 showed very little efficacy in 12 MCC patients [179].

1.7 Immune evasion of VP-MCC

More than 90% of the MCC patients are immunocompetent and VP-MCC tumors are highly antigenic, yet they evade immunological destruction [57,63,180]. MCPyV can escape detection by the immune system by different mechanisms. Down-regulating major histocompatibility complex class 1 (HLA class 1) was observed in 84% of MCC tumors, and HLA class 1 expression was significantly lower in VP-MCC than in VN-MCC [181]. MCPyV-specific T cells and MCC-infiltration lymphocytes express elevated levels of multiple markers of exhaustion such as programmed death 1 (PD-1) and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) [182]. Moreover, the level of vascular E-selectin is reduced in >50% of the examined MCC (n=56; viral status not determined) and this negatively affects the ability of lymphocytes to migrate into the tumor microenvironment [183]. Programmed death ligand -1 (PD-L1) may be aberrantly expressed by tumor cells, creating a shield against immune attack [184].

Immunohistochemical staining of 8 VN-MCC and 34 VP-MCC showed that none of the VN tumors expressed PD-L1, while 50% of the VP-MCCs were positive for PD-L1 [185]. Another study on 14 MCC (6 VN and 8 VP) reported that 1 VN-MCC had few (1%) PD-L1 positive tumors cells, whereas 7 of the VP-MCC were PD-L1 positive with 2-7.5% of the cells expressing PD-L1 [186]. It is not known whether MCPyV can affect the expression of PD-L1, but upregulation of PD-L1 has been observed in persistent infection with the oncoviruses hepatitis B and C [187].

MCPyV can also avoid the innate immune system because its early region downregulated the expression of TRL9 in the B lymphocyte RPMI-8226 cell line by targeting the transcription factor C/EBPβ [188]. sT could also reduce TLR9 expression, but the mechanism is not known, but it may operate by stabilizing LT [134]. A study on 128 MCC patients revealed that decreased expression of TLR 9 correlated strongly with MCPyV positivity of the tumor, while expression of TLR2, 4, 5, and 7 did not correlate with the viral status of the tumor [189].

The interference with the NFkB pathway by MCPyV sT was discussed earlier. However, another putative mechanism by which MCPyV can interfere with this pathway is through the interaction of LT with bromodomain protein 4 (Brd4). Brd4 acts as a transcriptional and epigenetic regulator [190], and can interfere with the NFκB pathway by interacting with IκB [191]. Brd4 stimulates MCPyV DNA replication by interacting with MCPyV LT and recruitment of replication factor C [113]. Arora *et al*. showed that also tLT binds Brd4 and that co-expression of Brdr4 in combination with either LT, sT, or tLT did not stimulated MCPyV promoter activity in U2-OS cells [114]. However, Brd4 in combination with LT+sT, but not with tLT+sT, enhanced promoter activity. Studies by our group showed that full-length LT inhibiting the activity of early as well late promoter from 8 different MCPyV variants in MCC13 and immortalized human dermal fibroblasts, whereas truncated variants stimulated their cognate promoter in both cell lines. The effect of sT on MCPyV promoter activity was not examined [25]. The study by Arora *and colleagues*

was done in U2-OS cells and it was not specified if early or late promoter activity was monitored and from which virus strain the promoter was derived. Moreover, they used tLT referred to as tLT21 and tLT168, while we used tumor-derived tLT and tested their effect on the corresponding promoter [114]. Whether the MCPyV LT:Brd4 interaction interferes with NF◎B signaling pathway and contributes the virus-induced tumorigenesis remains to be investigated.

Cytokines trigger inflammatory and immune responses upon viral infection [192,193], and play a pivotal role in tumorigenesis [194,195]. A study in BJ human foreskin fibroblasts showed that tLT or tLT+sT induced IL-1β, IL-6, IL-8, and CXCL1 levels, but their expression levels have not been monitored in VN- and VP-MCC cell lines or tumor tissue [196]. Prokineticins are chemokine-like proteins that possess angiogenic and immunoregulatory activities [197]. VP-MCCs had higher prokineticin-2 mRNA levels than the virus-negative tumors [198]. Our group found that chemokine (C-C motif) ligand 17/thymus and activation-regulated (CCL17/TARC) is upregulated in VP-MCC cell lines compared to VN-MCC cell lines. Full-length and tLT, but not sT, enhanced the CCL17/TARC promoter activity and increased protein levels [199]. The exact mechanisms by which MCPyV may affect cytokine expression and their possible role in MCC remain to be determined. Another study reported that sT downregulates IL2, IL-8, CCL20 and CXCL9 expression in the VN-MCC cell MCC-13 [119], but expression levels in VN- and VP-MCC tumors have not been compared. Stimulator of interferon genes (STING) is a signaling molecule that controls type I interferon and other pro-inflammatory cytokines production [200]. STING protein was undetectable in VP MKL-1, MKL-2 and MS-1 cells, but not in non-classical VN MCC13, MCC26 and UISO cells. Five MCC tumors (virus state not mentioned) also stained negative with STING antibodies [201]. STING silencing may help MCC tumor cells to escape immune eradication. More VN- and VP-MCC should be scrutinized to establish whether STING is specifically silenced in the VP-MCC and the potential role of T antigens in silencing STING should be explored.

1.8 Specific biomarker for VP-MCC

Apart from detection of viral DNA, RNA, and protein, diagnostic markers that specifically discriminate VP-MCC from VN-MCC are lacking. Likewise, biomarkers to predict disease progression and response to therapy of VP-MCC are lacking. However, the presence of antibodies against LT and sT may be used a diagnostic and prognostic marker. While most individuals have antibodies against MCPyV VP1 (see 1.2), only ~1% healthy patients had low titer antibodies against viral T antigens, whereas 41% of MCC patients had such antibodies [202]. The viral status of all MCC patients was not known, but for those patients it was known, serology for the LT/sT much more closely reflected the virus status of the tumor. In addition, the titers of T antigens antibodies decreased rapidly in patients whose cancer did not recur, whereas they rose with disease progression. So antibodies against LT/sT can predict if the patient has a VP tumor, but these antibodies can also be used to monitor the development of the disease and whether the patient respond to treatment or not.

Some putative markers will be discussed in this section, although most of them do not seem to be very specific and more VN- and VP-MCC patients need to be studied to validate their usefulness.

Several studies have shown that p63 may be an adverse prognostic factor as high levels have been linked to a worse prognosis [203-206], but the viral status in the MCC tumors was not always described. In one follow up study, the presence of MCPyV was examined, but no correlation between p63 expression and viral presence was found [207]. The chromatin architectural factor DEK was found to be expressed in 15/15 MCC tumors examined, but the viral presence or the clinical stage of the tumors was not identified [208]. This protein is also overexpressed in other cancers (reviewed in [209]), so that it is not a specific MCC biomarker. K homology domain-containing protein overexpressed in cancer (KOC=IMP3) is overexpressed in 90% of the MCC samples (n=20) and expression correlated with metastasis, but the relationship with MCPyV was not investigated [210]. KOC is a prognostic

marker in pancreatic cancers and melanomas [211,212] and might be a prognostic marker for MCC. Other proteins examined in MCC include vitamin D receptor, the inhibitory ligand of the Notch receptor Delta-like protein 3 (DDL3), HIF-1◎ and its target genes GLUT-1, MCT4, CAIX, and vascular endothelial growth factor receptor 3 (VEGFR-3), and P-cadherin [213-219]. However, the viral status of the tumor was not known (vitamin D receptor), no difference between VN- and VP-MCC was found (GLUT-1, MCT4, CAIX), or there was a tendency to higher expression in VP-MCC, but the difference was not significant (DDL3, HIF1◎, P-cadherin). VEGFR-3 was found in all MCCs, but significantly higher in VP-MCC [219]. The value of VEGFR3 as a biomarker is controversial because other studies failed to detect VEGFR-3 in MCC [213,214]. The inconsistency, lack of virus status and limited number of samples of these studies have failed to identify a bona fide biomarker for VP-MCC.

MicroRNA as VP-MCC biomarkers

MicroRNA (miRNA) are small RNA molecules that inhibit gene expression at a post-transcriptional level by preventing translation or inducing degrading of their target mRNA. Because of their stability, presence in all body fluids, and sometimes disease-specific expression, they can be useful prognostic and diagnostic markers in cancer. Several groups have examined miRNA expression in MCC (reviewed in [220]), but miRNA-375 in particular has been more extensively studied. This miRNA (mcv-miRNA-M1) is complementary to a sequence in the *LT* gene adjacent to the RB binding motif. However, mcv-miRNA-M1 was detected in ~50% of 38 tested VP-MCC and the expression levels were low [221]. It seems unlikely that mcv-miRNA-M1 contributes to MCVPyV-induced tumorigenesis and its use as biomarker for VP-MCC is doubtful.

1.9 MCPyV in *non*-MCC tumors

Considering the role of MCPyV in the development of MCC and the widespread prevalence of the virus across the body led researchers to investigate a possible involvement and role of MCPyV in *non*-MCC cancers. In most of the *non*-MCC tumors studied, MCPyV DNA was investigated by PCR. However, in those cases in which the copy number of the viral genome/cell was analyzed, MCPyV copies were very low with <<1 copy/cell, that is, several logs lower compared to VP-MCPyV tumor cells. In those cases, in which the expression of LT was monitored by immunohistochemistry, LT could only be detected in a handful of cases, even though the viral DNA was present. The earliest observation associating MCPyV with *non*-MCC was the detection of MCPyV DNA in non-melanoma cancers of the skin from immunocompromised patients [222]. MCPyV was detected in many other neoplasia also in non-immunocompromised individuals. Among the different body sites, the integumentary system is represented as a site for MCPyV-positive *non*-MCC tumors. Many non-melanoma skin cancers, including squamous cell carcinomas [222-228] and basal cell carcinomas [222,229-231], are frequently found to harbor MCPyV DNA or transcripts. The presence of the virus was also detected in cases of keratoacanthoma [224,229,232], Kaposi's sarcoma [233,234], porocarcinoma [226,235], atypical fibroxanthoma [229], and Langerhans cell sarcoma [236]. On the contrary, melanomas are not associated with MCPyV [237-239], except for one case in which MCPyV LT transcripts were detected in acral lentiginous melanomas and in nodular melanomas, whereas superficial spreading melanomas were virus-negative [230]. The viral load of MCPyV was significantly higher in these skin-related samples compared to other virus-positive *non*-MCC cancers, but the expression of the viral LT was detected in only one case of a combined MCC–squamous cell carcinoma [240]. The high viral load observed in skin-related *non*-MCC cancers might not be a surprising event given a close proximity to the original replication of the virus would render other cells of the skin susceptible to the presence of the virus. Compared to skin related *non*-MCC tissue,

tumors of the circulatory system do not have a higher frequency of MCPyV transcript-positive tissues or a higher rate of genomic integration, while a full LT protein expression could be detected in some of the tumors. MCPyV prevalence is low in most of the lymphatic system cancers studied, except for tonsillar squamous cell carcinoma [241,242] and thymoma [243-245]. The expression of the LT was examined in thymomas and from the seven samples that were MCPyV DNA-positive, with three containing detectable LT protein levels [244]. It is important to note that benign lymph nodes also contained the transcript of the MCPyV sT [245] and that the genome copy number in all positive examined lymphatic system tumors ranged between 0.000004 and 0.0013 [241,242]. MCPyV also exhibited a presence in tumors of the circulatory system, as many leukemia cells were found to contain MCPyV sequences. One acute myeloid leukemia sample was positive for MCPyV DNA [246], although, in other cases, no sign of the virus was observed [247] Chronic lymphocytic leukemia also contained MCPyV transcripts [248-251], whereas truncated LT mRNA was also detected in samples of which two also harbored full-length LT mRNA [249]. When examined, all the chronic lymphocytic leukemia were negative for LT protein expression [21,252]. The detection of the truncated LT transcripts is considered a sign of viral genomic integration, however, considering that some samples also contained full-length LT, it is plausible that in these tumors the virus was firstly present in the cytoplasm before integration and later lost its ability to express its proteins. Cutaneous T-cell lymphoma (CTCL) is a special non-Hodgkin's lymphoma, which is migrating to and resides in the skin. Therefore, it is potentially more exposed to MCPyV than other leukemia cells. Studies on the association between MCPyV and CTCL would seem conflicting since some are reporting no detectable levels of MCPyV in CTCL cells [253-255], while others described a certain level of MCPyV DNA and transcripts in CTCL [256,257]. When the CTCL containing skin lesions were examined together with neighboring non-lesioned skin tissues, the MCPyV prevalence was similar [258,259]. However, it is important to note that one of these studies has detected the expression of the VP1

protein in four and the expression of LT protein in one CTCL-related samples, which were completely absent in the controls [258]. Cutaneous B-cell lymphomas (CBCL) are skin-resident, generally slowly growing B-cell lymphomas. Considering the fact that these cells are also in close proximity to the viral replication sites at the skin, the potential contribution of the virus to the development of CBCL cannot be ruled out. Yet, the available studies were only able to detect the MCPyV in CBCLs with a relatively low prevalence, without viral protein expression [21,257,260], and with a low viral load [257]. No presence of MCPyV was detected in the studies examining chronic myelomonocytic leukemia cells [247], mantle cell lymphoma cells [261], follicular lymphomas [262], primary effusion lymphomas [233], small-cell carcinomas of the lymph nodes [263], and acute lymphoblastic leukemia [264]. LT transcripts were found in one study, analyzing a set of non-Hodgkin's lymphomas [265]. The available reports regarding the presence of MCPyV DNA in reproductive system-related tumors are scarce, although some studies are displaying some level of occurrence of the virus in prostate cancer [266,267], breast cancer [268], and cervical cancer [269]. In one case of testicular cancer, the viral load was relatively high at 0.934 copies/cell even if LT protein expression was not assessed [266]. MCPyV-positive prostate cancers showed a lower viral copy number [266], with samples from cervical cancer containing an even lower level of viral copies [269,270]. MCPyV viral transcripts were not detected in any examined ovarian cancer and in the cancers of the vulva [271,272] and only a small set of breast cancer cells were shown to contain viral transcripts [268,271]. Despite the low viral genome copy number, LT transcripts and protein were detected in HIV-positive women affected by cervical cancers [270]. It is possible that the expression of LT in these tumors will have originally resulted from an HIV-related condition. Tumor samples originating from the digestive track are harboring the MCPyV sequences with a higher frequency as in the case of esophagus cancer (45.1%) [273], liver cancer (62%) [266], or salivary gland cancer (26.2%) [274]. Only one tumor sample, a small-cell carcinoma of the parotid showed expression of LT protein [275] in which case, a LT

truncating mutation was also found [275]. There was a significant difference between squamous cell carcinomas of the oral cavity and other oral cavity tumors, since approximately 40% of the examined oral cavity squamous cell carcinomas were positive for viral transcripts [266] while these were barely detectable in other tumors. Sporadically tumors of the larynx, tumors of the mandible, throat, tongue [276] and tumor of the jaw [265] were MCPyV DNA-positive, but it is important to note that healthy oral tissues contained MCPyV transcripts at a relatively higher frequency of approximately 17% [277]. LT protein was not detected in either of them and, the copy number of the virus per cell, was low [265]. Many other examined digestive track-associated cancers, including stomach cancer [271,278] and colorectal cancer [222,278-280], showed a very low or no positivity to viral transcripts. It is possible to speculate that the virus is present in the proximal part of the digestive system at a low level but not in other parts. A low prevalence of MCPyV was detected in bladder [266,271,278] and renal cancers with the viral load in the tumor cells relatively low compared to MCCs [266]. Considering that none of these tumors had a detectable level of LT protein [271,278], it is possible to conclude that MCPyV does not play a causative role in these cancers. MCPyV was observed in the urine of healthy patients [281], although, until now, it is not clear whether this represents a way the body clears out the virus or whether these viral particles are originating form a potential host cell in the excretory system. MCPyV has sporadically been identified in tumors originating from other organ systems. MCPyV can rarely be traced in tumors of the skeletal system, including Ewing sarcomas, chordomas, chondrosarcoma, and rhabdosarcomas [272,282]. A limited number of desmoplastic tumors are the only soft tissue-related tumors examined thus far and did not harbor MCPyV DNA [272]. Studies focusing on tumors of the nervous system described a limited number of cases in which MCPyV transcripts were detectable in a few schwannomas, meningiomas, glioblastomas [283], and neurofibromas [265], whereas no relation to the virus was established in neuroblastomas [271,272,284]. Neuroblastoma is a childhood cancer, with approximately 90% of the cases

occurring in children less than 5 years old [285]. At this early age, children may not yet have been infected with MCPyV because sero-epidemiological studies demonstrated that the prevalence of MCPyV in a specific tumor sometimes varied from study to study.

As previously mentioned, MCPyV can also replicate in cultures of lung fibroblasts [286]. Therefore, it might not be unexpected to detect MCPyV in respiratory system and in tumors of this body compartment. Based on the histological similarities between MCC and pulmonary neuroendocrine carcinoma (NEC), including SCLC and large-cell NEC (LCNEC), the possible association between MCPyV and pulmonary NEC has been studied. Two German groups showed the presence of MCPyV DNA in a subset of SCLCs, with detection rates of 6.7% (2 out of 30) and 38.9% (7 out of 18) [287,288]. In contrast, no evidence of a causal relationship between MCPyV and pulmonary LCNEC has been reported [289-291]. North and South American groups detected MCPyV DNA in 16.7% (5 out of 30) and 4.7% (4 out of 86) of NSCLCs, respectively [292,293]. In these studies, a general lack of viral presence in these tumors was demonstrated [278,294,295] and, it is worth mentioning that healthy lung tissues are not well studied in this respect [233,266,294-296]. MCPyV prevalence in non-small cell lung cancers (NSCLCs) has not been investigated well. NSCLC was associated with the presence of MCPyV [243,297,298], although none of the examined NSCLC samples showed any LT protein expression [299]. An exception was the detection of truncated LT in two NSCLC [243]. In one of these two NSCLC, a sample displayed both episomal and integrated MCPyV DNA and the expression of both the full-length and truncated LT protein [243]. It is possible to highlight the possibility that the episomal MCPyV genome could be maintained separately in the cytoplasm despite the integration to the genome, as similarly observed in a few MCCs [300,301]. Another study showed the LT expressed in NSCLC [302]. It is feasible to hypothesize that, this high number of LT expressing lung carcinoma cells are arising from the fact that the virus could potentially propagate in the vicinity of these cells and, therefore it could potentially

integrate to the lung-related cancer cells. This hypothesis should be studied more thoroughly.

1.10 MCPyV in *non*-cancerous tissues

MCPyV DNA has been detected in various non-cancerous tissues of the body like the adrenal gland, spleen, bone mar\row, stomach, gallbladder, pancreas, heart, and aorta, although with a relatively low viral load [303]. On average, the viral genome copy number of MCPyV was 60 times lower in healthy tissues across the body compared to MCC samples [266]. A study identified the highest detection of MCPyV among different tissues in the digestive system, saliva, and in the upper digestive tract [266]. MCPyV DNA was also present in bodily fluids, such as the blood and urine from two patients with advanced MCC. However, viral DNA was not detected in the whole blood samples of patients without MCC [304]. MCPyV positivity in blood was associated with monocytes, and MCPyV was shown to be selectively associated with the CD14+ CD16 'inflammatory' monocyte subpopulation. This finding suggests that inflammation-associated monocytes might serve as potential vehicles for MCPyV, which could aid viral transmission in the body through harboring and transferring the virus to inflammation sites [304]. In contrast, MCPyV DNA sequences were amplified in the buffy coats of blood, with a very low viral load of 10 to 100 molecules/100 000 cells [305]. The low levels of MCPyV DNA could be attributable to the fact that the blood samples were pre-fractionated by a density gradient centrifugation in order to obtain the leukocyte rich fraction. Subsequently, circulating MCPyV DNA was detected in the unfractionated sera of healthy individuals by using a more sensitive quantitative PCR and droplet digital PCR, with the prevalence of 2.6% and a low viral load of 1–5 copies/lL [306]. The presence of MCPyV VP1 transcripts was also detected in the urine of both immunosuppressed and non-immunosuppressed individuals [281]. This data suggests that MCPyV could potentially be transferred to many tissues through bodily fluids. It is clear that, since the viral load in these compartments is low, MCPyV is most likely just

passively being transferred and actively replicating not in blood cells the bloodstream nor in epithelial cells in urine.

2. AIMS:

That MCPyV is the causative agent of MCC it has been widely demonstrated. However, the prevalence of MCPyV in *non*-MCC population and its possible role in the pathogenesis of other cancers are not fully known yet.

As previously discussed, MCPyV DNA fragments have been detected in a wide variety of anatomical sites, including the upper and lower respiratory tract samples of individuals with variable respiratory symptoms [307-313] including patients presenting abnormality or impairment of the immune system or subjects suffering from chronic disease such as, Cystic Fibrosis (CF).

In recurrent pulmonary exacerbation, the role of chronic bacterial infections is well-known, whereas the role of viral infections is still debated [314] and deserves to be explored. Common viral infectious agents in CF individuals include Rhinovirus (HRV) [315], Respiratory syncytial virus (RSV), and Influenza A and B viruses [316,317]

To date, only a few two studies examined the prevalence of MCPyV infection in CF patients reporting different frequency values (6.8% [311] and 26% [313]) and raising questions about the MCPyV immunological and clinical relevance in the respiratory tract.

Upon infection, HPyVs dsDNA can be sensed by Toll-like receptor 9 (TLR9), a key receptor in the host innate immune response that recognizes viral or bacterial dsDNA in the form of non-methylated CpG motifs [318]. Upon ligand binding, TLR9 induces the transcription nuclear factor NF-kappaB (NF-kB), leading to increased production of inflammatory mediators and interferon (IFN) [319]. In order to escape from immune recognition responses, MCPyV, and other dsDNA oncogenic viruses, have developed evasion strategies to alter the production of TLR9. The expression of the MCPyV LT can downregulate TLR9 expression in epithelial and MCC-derived cells [320]. Moreover, it has been found that the antiviral and antitumor actions of

IFNβ can be suppressed in cells transformed by wild-type MCPyV through a JAK1-MCPyV LT antigen interaction [321]. The antagonistic relationship between HPyVs and type I IFN (IFN–I) is also confirmed by studies evaluating HPyVs sensitivity to IFN-I action [322]. In particular, *in vitro* and *in vivo* experiments carried out in MCPyV infected MCC cell lines and mice respectively, showed that IFN-I modulated LT activity promoting the expression of promyelocytic leukemia protein, which interferes with the function of the LT [323].

In this study, as objective one, the prevalence of MCPyV-DNA in respiratory samples of a large cohort of CF patients was estimated analyzing viral load and sequencing the viral early region LT, the NCCR and the late region corresponding to VP1, from all MCPyV-DNA positive respiratory samples.

In addition, in order to shed light on the potential pathogenic role of MCPyV in CF, demographic, microbiological and clinical data collected from MCPyV-DNA positive and negative patients were compared.

Given that TLR9, through the binding of intracellular viral DNA, plays a major role in promoting the activation of antiviral pathways associated to IFN-I response [319,324], hypothesizing that alterations in the expression of TLR9 in the respiratory tract of MCPyV positive CF patients might lead to attenuated antiviral innate immune responses, the transcript expression of TLR9 and distinct IFN-I genes (IFNα, IFNβ and IFNε) were examined in respiratory samples of MCPyV positive CF patients according to their bacteriological and clinical status.

As second objective of the study, the role of MCPyV as etiological viral agent of NSCLCs was thorough by examining a series of NSCLC-patients for both the presence of specific MCPyV DNA and the expressions of viral RNA transcripts and virally encoded protein. The integrated form of the MCPyV-positive NSCLCs was also examined.

The v-raf murine sarcoma viral oncogene homolog B1 (BRAF) gene expression was found to be higher in MCPyV positive samples than negative ones [325]. Since BRAF is a downstream target of epidermal growth factor receptor (EGFR) pathway, activated EGFR could increase BRAF expression, suggesting a potential association between MCPyV infection and EGFR activated mutations. Furthermore, since MCPyV itself undergoes specific mutations during MCC tumorigenesis, it has been hypothesized that MCPyV might be one inducer of EGFR mutations in NSCLC. For these reasons, in this study, MCPyV infection and the EGFR mutations was also screened.

3. MATERIALS AND METHODS

Objective one:

3.1 Study population

Respiratory samples [n = 1138 (oropharyngeal aspirate n = 619 and sputum samples n = 519)] were randomly collected from CF patients (n = 539) attending the Lazio Regional Reference Center for CF, Policlinico Umberto I Hospital, Sapienza University of Rome, for routine visits during July 2018–October 2019. The type of respiratory sample, oropharyngeal aspirate or sputum, did not differ with respect to CF patients' age (age of patients with oropharyngeal aspirate samples: mean/standard deviation, 25.4/16.5 years; age of patients with sputum samples: mean/standard deviation, 25.4/16.5 years, $p > 0.05$). Although follow-up schedules can change depending on the health status of CF patients, most individuals were routinely seen in the hospital every 3 months: the median follow-up period was 2.8 months (range: 0.4–15.4 months). Moreover, one respiratory sample was collected from 173 (173/539, 32.1%), two respiratory samples from 197 (197/539, 36.5%), three respiratory samples from 105 (105/539 19.5%) and four respiratory samples from 64 (64/539, 11.9%) out of 539 CF patients, respectively. Demographic and clinical data, such as gender, age, genotype, BMI, pulmonary function, exacerbation events were collected from medical records. Following the usual microbiological investigation, MCPyV-DNA was tested by real time PCR in the residual respiratory samples. Then, the gene expression analysis of TLR9 and IFNα, IFNβ, IFNε was carried out in a subgroup of 95 MCPyV-DNA positive samples and 147 negative samples of CF patients for whom the respiratory specimens were enough to allow real-time RT-PCR assays. The study was approved by the local Ethic Committee (Ethic Committee Sapienza University of Rome, Policlinico Umberto I Hospital) N 5223 (approval date 25 October 2018), and informed consent was obtained from patients or their parents when necessary.

3.2 Microbiological investigations

Respiratory tract samples were analyzed for common microorganisms by standard methods and procedures for the traditional microbiological investigation (BD BBLTM Stacker Plates, Heidelberg, Germany). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Inc., Billerica, MA, USA) was used for bacterial identification where necessary. Since *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most prevalent bacterial species involved in respiratory tract infection in CF, patients were stratified according with the presence of these bacteria for the viral and immunological analysis.

3.3 MCPyV DNA extraction and quantification by real-time PCR

Total DNA was extracted from respiratory samples using the DNeasy® Blood & Tissue Kit (QIAGEN, Milan, Italy), according to the manufacturer's instructions. Specific real-time PCR assays were performed using TaqMan-based quantitative PCR, employing primers and probes targeting MCPyV sT, as previously described [313]. All samples were tested in triplicate, and the number of viral copies was calculated from standard curves constructed using a ten-fold dilution series of plasmid pMCV-R17a containing the entire genome of MCPyV (Addgene, #24729) (dilution range: 10^8–10 copies/mL). The lower detection limit of the assay was 10 DNA copies of the target gene per amplification reaction, corresponding to 10 copies per reaction (10 copies/reaction). The amount of cellular DNA was quantified simultaneously using a SYBR Green PCR for the housekeeping b-globin gene and used to normalize the MCPyV DNA.

3.4 Standard PCR for NCCR, LT and VP1 sequences

MCPyV-positive DNA samples were subjected to nested PCR for the amplification of the NCCR region. Two sets of primers, ORIF1/ORIR1 and ORIF2/ORIR2, were employed to generate an NCCR fragment of 504 and 203 base pair (bp). PCR

reactions were carried out following a published protocol [326]. To amplify the MCPyV LT and VP1 sequences, three primer sets, LT1, LT3, and VP1, were used to generate a LT amplicon with sizes of 440 and 309 bp, respectively and a VP1 fragment of 351 bp [62]. The b-globin gene was amplified to confirm the presence of PCR-amplifiable DNA. Water containing all the PCR components except template DNA was used as the PCR-negative control. The PCR products were then separated electrophoretically on 2.0% agarose gels, visualized with ethidium bromide staining, and observed under UV. The PCR products were purified with miPCR purification kit (Metabion, Plannegg, Germany) and sequenced in a dedicated facility (Bio-Fab research, Rome, Italy). DNA sequencing was performed with a Sanger protocol (Big Dye Terminator Sequencing, Life Technologies), using an ABI 3730 System (Life Technologies, BioFab research s.r.l., Rome, Italy). The obtained sequences were compared to the reference strain MCC350, EU375803 strain and sequence alignment was performed using ClustalW2 [http://www.ebi.ac.uk/Tools/msa/clustalw2/] available on the EMBL-EBI website using default parameters.

3.5 TaqMan-based real-time RT-PCR assays for mRNA expression

The mRNA levels of TLR9 (Hs.PT.58.40576968, Integrated DNA Technologies, IDT, Coralville, IA, USA), IFNα (Hs.PT.58.24294810.g, IDT), IFNβ (Hs.PT.58.39481063.g, IDT), and IFNε (Hs.PT.58.4812867.g, IDT) were measured by quantitative real-time RT-PCR assays carried out with the LightCycler 480 instrument (Roche, Basel, Switzerland) as previously described [329]. Briefly, total RNA was extracted from respiratory samples using the RNeasy Plus Universal Tissue Mini Kit (Invi- trogen, Carlsbad, CA, USA) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to the manufacturer's protocol. Primers and probes for each gene were added to the Probes Master Mix (Roche, Basel, Switzerland) at 500 and 250 nM, respectively, in a final volume of 20 μL. The house- keeping gene β-glucuronidase/GUS was used as an internal control.

Gene expression values were calculated by the comparative cycle threshold value (Ct) method (2^-ΔCt).

3.6 Statistical methods

Data analysis involved a process of indexing, coding, and data cat- egorizing. All statistical analyses were performed with Statistical Pack- age for Social Science (SPSS) version 25, which is a graphical user interface for MATLAB (ver. 2021). Categorical variables were summarized as proportion, absolute frequency (and/or percentage), and continuous variables were summarized as mean and Standard Deviation (±SD) or median and inter-quartile range (IQR: 25th and 75th percentile). Baseline demographic and outcome variables were compared to the presence/absence of MCPyV-DNA and exacerbation using Chi- square ($\chi 2$). Differences in the levels of TLR9 and IFNs between MCPyV-DNA positive and negative CF patients were evaluated using the Mann–Whitney test. The same test was used to compare the levels of mRNA of TLR/IFNs between MCPyV-DNA positive and negative CF patients according to the microbiological status. Spearman's rho coefficient (r) was calculated to assess the correlation between TLR9 and IFNs levels, and age. A series of repeated measures analysis of variance (RM-ANOVA) were carried out to assess whether there was a difference in viral load over four assessment time points (baseline, T1, T2 and T3 follow-up period). RM-ANOVA F-scores at p-value less than 0.05 for each subscale were considered significant. Intervention was compared on baseline characteristics as well as baseline measures of the outcomes. Logistic regression analysis was fitted to develop models of risk factors for exacerbation occurrence in MCPyV-DNA positive CF patients.

Objective two:

3.7 Patients and samples

Formalin-fixed paraffin-embedded tissue (FFPE) of primary resectable NSCLC retrieved from the archived specimens of the Policlinico Umberto I—Sapienza University of Rome (Rome) were obtained from 112 Italian patients. Specifically, out of 112 NSCLC, 32 were squamous cell carcinomas (SCCs; denoted SCC 1–32), 45 adenocarcinomas (AC 1–45) and 32 large-cell carcinomas and 3 pleomorphic carcinomas (PL1– 3). The median ages of the patients were 70 years for SCCs, 65 years for ACs, 73 years for large-cell carcinoma, and 70 years for PL. There were 25 male and 7 female SCCs patients, 24 male and 21 female ACs patients, 28 male and 4 female LCCs patients, and 3 male PL patients. Of the 112 lung cancer patients, 80 were smokers and 32 were never smokers.

3.8 Detection of MCPyV with standard PCR

DNA was isolated with the DNeasy Blood and Tissue Kit (QIAGEN, Milan, Italy). To detect the MCPyV LT and VP1 genes, three primer sets, LT1, LT3, and VP1, were used as described previously [62]. The b-globin gene was amplified to confirm the presence of PCR-amplifiable DNA. Water containing all the PCR components except template DNA was used as the PCR-negative control. The reaction conditions included denaturation at 95 °C for 10 min, followed by amplification with 40 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s, with a final extension for 7 min at 72°C. The PCR products were then separated electrophoretically on 2.0% agarose gels, visualized with ethidium bromide staining, and observed under UV light. After purification of the PCR products by the MinElute PCR Purification Kit (QIAGEN, Milan, Italy), sequencing was performed in a dedicated facility (Bio-Fab Research, Roma, Italy). The obtained sequences were compared to reference sequences deposited in GenBank. Sequence alignments were performed with ClustalW2 at the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-

EBI) website using default parameters [ClustalW2–Multiple Sequence Alignment. Available online: http://www.ebi.ac.uk/Tools/msa/clustalw2/].

3.9 Quantitative real-time PCR (qPCR) for MCPyV sT

The standard PCR-positive samples were subjected to a TaqMan-based quantitative PCR (qPCR), employing primers and probes targeting MCPyV sT, as reported below [313]. The reaction conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were tested in triplicate, and the number of viral copies was calculated from standard curves constructed using a ten-fold dilution series of plasmid pMCV-R17a containing the entire genome of MCPyV (Addgene, #24729) (dilution range: 10^8–10 copies/mL). The lower detection limit of the assay was 10 DNA copies of the target gene per amplification reaction, corresponding to 10 copies per reaction (10 copies/reaction). The amount of cellular DNA was quantified simultaneously using a SYBR Green PCR for the housekeeping b-globin gene and used to normalize the MCPyV DNA.

3.10 Reverse transcription–PCR (RT-PCR)

Total RNA was extracted from the FFPE specimens with the Quick-RNA Miniprep Plus Kit (Zymo Research). The total RNA was treated with DNase to avoid the amplification of viral DNA. The RNA (1 mg) was reverse transcribed using the Zymoscript RT PreMix Kit (Zymo Research). An aliquot (1 ml) of the reverse transcription reaction mixture was used for the subsequent PCR amplification. The b-globin gene was amplified to confirm the presence of PCR- amplifiable cDNA.

3.11 Sequencing analysis of the MCPyV LT gene

The DNA sequences of the viral genomes from nucleotide positions 151–3102 (based on Genbank strain EU375803), which include the entire LT gene, were determined by PCR using different combinations of six primer sets. This was followed by a direct sequence analysis of the amplified products.

3.12 Analysis of the MCPyV integration sites

The integration sites of MCPyV were investigated using the detection of integrated papilloma sequences (DIPS)–PCR technique, as described previously [272]. This method allows the amplification of the junctions between viral and cellular genomes [327]. Briefly, after the DNA was digested with the Taq I restriction enzyme, the DNA fragments obtained were ligated to enzyme-specific adaptors. The ligated fragments were subjected to PCR amplification using viral- and adaptor-specific primers. The PCR products were purified and sequenced as described above. The integration sites were determined by submitting the sequences to the databases of the National Center for Biotechnology Information and analyzing them with the Basic Local Alignment Search Tool (BLAST) for genomic localization.

3.13 Analysis of EGFR mutations

Exons 19, 20 and 21 of the EGFR gene were amplified by PCR as described previously [328]. PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide to confirm the size of the bands. DNA sequencing for EGFR mutations was then performed by dedicated facility (Bio-Fab Research, Roma, Italy). All sequencing reactions were performed in both the sense and antisense directions. DNA isolated from normal lung tissue was also amplified and sequenced to verify the EGFR alteration.

3.14 Statistical methods

The statistical correlations between the variables were analyzed with Fisher's exact test. A p-value less than 0.05 were considered significant.

4. RESULTS

Objective one:

4.1 MCPyV prevalence, viral load and seasonality

One thousand hundred thirty-eight respiratory samples were randomly collected from 539 CF patients and analyzed for MCPyV-DNA. Overall, 137/539 (25.4%) CF patients were positive at least one time (**Table 3**), for a total of 268/1138 MCPyV-DNA positive respiratory specimens (23.5%) (**Table 4**).

Table 3. Merkel cell polyomavirus (MCPyV) prevalence in cystic fibrosis (CF) patients (n=539). Demographic, clinical characteristics of Merkel cell polyomavirus (MCPyV) positive (n=137) and negative (n=402) cystic fibrosis patients.

Items	Total patients n=539	MCPyV-DNA (+) patients n= 137*	MCPyV-DNA (-) patients n=402
Respiratory samples			
One sample (%)	173 (32.1)	17 (12.4)	156 (38.8)
Two samples (%)	197 (36.5)	45 (32.8)	152 (37.8)
Three samples (%)	105 (19.5)	40 (29.2)	65 (16.2)
Four samples (%)	64 (11.9)	35 (25.6)	29 (7.2)
At least one positive sample (%)	137 (25.4)	137 (100)	NA
At least two positive samples (%)	82 (15.2)	82 (59.9)	NA
At least three positive samples (%)	36 (6.7)	36 (26.3)	NA
Four positive samples (%)	13 (2.4)	13 (9.5)	NA
Two consecutive positive samples (%)	50 (9.3)	50 (36.5)	NA
Three consecutive positive samples (%)	19 (3.5)	19 (13.9)	NA
Four consecutive positive samples (%)	13 (2.4)	13 (9.5)	NA
Male patients, n (%)	273 (50.6)	68 (49.6)	205 (50.9)
Age, mean (SD)	25.9 (16.5)	23.7 (15.4)	26.5 (16.8)
0-10 years	114 (21.1)	33 (24.1)	81 (20.2)

11-25 years	167 (31.0)	43 (31.4)	124 (30.8)
>25 years	258 (47.9)	61 (44.5)	197 (49.0)
ΔF508 homozygous/ heterozygous/others patients, n (%)	82 (17.6) / 211 (45.3) / 173 (37.1)	21 (16.7) / 51 (40.5) / 54 (42.8)	61 (17.9) / 160 (47.1) / 119 (35.0)
BMI, mean (SD)	20.5 (4.1)	19.9 (3.2)	20.7 (4.4)

*Patients positive for MCPyV-DNA detection in respiratory samples at least one time. Data are presented as number or mean. N.A.: not applicable.

Table 4. Demographic, microbiological and clinical features of Merkel cell polyomavirus (MCPyV) positive (n=268) and negative (n=870) cystic fibrosis (CF) samples divided by three age groups (0-10 years, 11-24 years, >25 years).

Items	MCPyV-DNA (+) samples (n=268)			MCPyV-DNA (-) samples (n=870)		
	0-10 years (n=74)	11-24 years (n=75)	>24 years (n=119)	0-10 years (n=182)	11-24 years (n=271)	>24 years (n=417)
Aspirate samples, n (%)	64 (86.5)	44 (58.7)	54 (45.4)	165 (90.7)	177 (65.3)	115 (27.6)
Sputum samples, n (%)	10 (13.5)	31 (41.3)	65 (54.6)	17 (9.3)	94 (34.7)	302 (72.4)
MCPyV titer, mean	$7.3*10^3$	$6.9*10^3$	$9.5*10^3$			
P. aeruginosa, n (%)	2 (2.7)	7 (9.3)	52 (43.7)	13 (7.1)	23 (8.5)	171 (41.0)
S. aureus, n (%)	41 (55.4)	42 (56.0)	20 (16.8)	84 (46.1)	137 (50.5)	73 (17.5)
P. aeruginosa and *S. aureus*, n (%)	7 (9.45)	9 (12)	32 (26.9)	23 (12.6)	61 (22.5)	95 (22.8)
P. aeruginosa mucoid, n (%)	2 (2.7)	7 (9.3)	62 (52.1)	4 (2.2)	42 (15.5)	170 (40.8)

Data are presented as number or mean. MCPyV titer is expressed as number of copies/ml.

Considering CF individuals with at least one follow-up sample (at least two samples), 82/539 (15.2%) patients were MCPyV-DNA positive at least two times;

36/539 (6.7%) patients were MCPyV-DNA positive at least three times; 13/539 patients (2.4%) had four MCPyV-DNA positive samples (**Table 3**). As far as MCPyV-DNA persistence in respiratory samples was concerned, 50 CF patients were positive in two consecutive samples (median follow-up period: 4.67 months, range: 0.7–10.2), 19 CF patients were positive in three consecutive samples (median follow-up period: 6.8 months, range: 2.8–11.4), 13 CF patients were positive in four consecutive respiratory samples (median follow-up period: 8.8 months, range: 2.2–11.8). Moreover, no statistically significant differences were found in the prevalence of MCPyV infection and in the levels of MCPyV-DNA between females and males and also analyzing CF patients stratified in three age groups (0–10 years, 11–24 years, ≥25 years) (**Table 3**). Analysis by qPCR showed a MCPyV-DNA titer in the respiratory samples ranged from 9×10^1 to 1.2×10^5 copies/mL, mean value of 8.2×10^3 copies/mL (± SD 7.8×10^3) and median Ct value of 30.2 (IQR: 28.1–32.2). There was no difference in MCPyV-DNA comparing aspirate and sputum samples in CF patients and between different age groups ($p > 0.05$ for all the analysis) (**Table 4**). Considering CF patients who remained positive for MCPyV-DNA detection in four consecutive respiratory specimens (n = 13, median follow-up period: 8.8 months, range: 2.2–11.8), by carrying out repeated MCPyV-DNA measures at different time intervals (T0-T1: 3.5 months; T1-T2: 2.3 months; T2-T3: 2.4 months), we found that the number MCPyV-DNA copies/mL decreased over the time ($p < 0.001$). Then, we evaluated the trend of monthly positivity rates of MCPyV-DNA in CF patients during the year analyzed (July 2018–October 2019). MCPyV-DNA was detected every month, with the greatest number of MCPyV positive respiratory samples found in February, October and December. The highest MCPyV prevalence was observed in February (18.2% of the month's samples), suggesting a winter seasonality for this DNA virus (data not shown).

4.2. MCPyV NCCR, LT and VP1 sequence analysis

Analysis of MCPyV NCCR regions obtained from the total of the positive MCPyV respiratory samples of CF subjects was carried out. The amplified NCCRs, spanning from nucleotide position 5077 to 5280, were compared with the reference sequence of the prototype North American strain MCC350, strain EU375803 [62] (**Figure 2**).

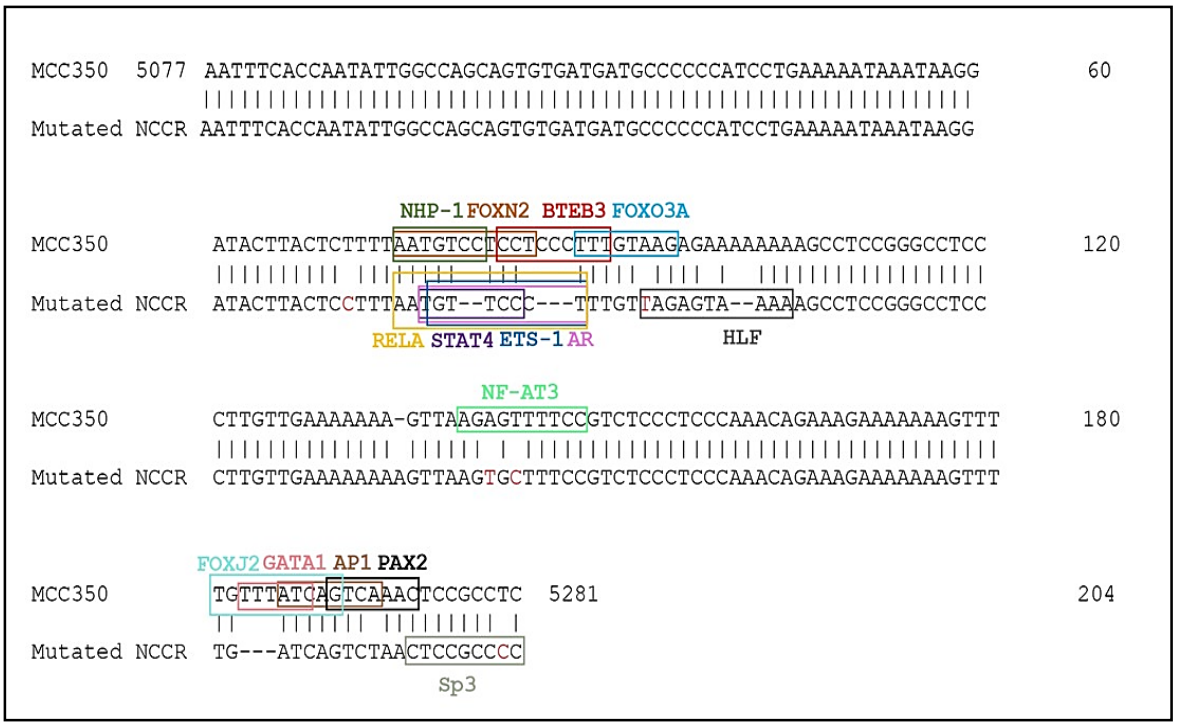


Figure 2. Sequence analysis of the MCPyV NCCR PCR products. The alignment is shown between the nucleotide sequence from 5077 (proximal to the early genes) to 5280 (just upstream of the start codon of the VP2 gene) of the published sequence of MCPyV in GenBank (NCBI) MCC350, strain EU375803 [62] and that obtained from the sequencing of positive samples containing the observed mutations and deletions. Putative binding sites for transcriptional factors in the MCC350, strain EU375803 [62] are reported as well as the changed-putative binding motifs resulted from the mutated NCCR.

Results showed a NCCR characterized by a high degree of homology with the prototype strain, despite the presence of some deletions, insertions, or mutations. Overall, a deletion of 3 bp (CCC), 3 TTT, 2 bp (CC) and 2 bp (AA), was frequently observed sited in positions 5163–65, 5260–62, 5157–58 and 5178–79, respectively (**Figure 2**). Interestingly, T to C transitions were recurrently observed in positions 5148, 5162, 5220 and 5280 (**Figure 2**); instead, A to T transversions were found in

position 5171, 5176, 5218 and 5270. Finally, a GTTGA insertion at positions 5210–5211 was identified. Analysis of putative binding sites was also carried. The NCCR sequence of the prototype MCC350, strain EU375803 [62] contains binding sites transcriptional factors such as NHP-1, FOXN2, BTEB3, FOXO3, NF-AT3, FOXJ2, GATA1, AP1 and PAX2 (**Figure 2**). Comparison of the cellular transcription binding motifs reported above with all the NCCR sequences recovered from MCPyV-DNA positive samples, evidenced that mutations, deletions, and insertions changed some putative binding sites in RELA, STAT4, ETS-1, AR, HLF and Sp3 as reported in **Figure 2**.

LT and VP1 sequencing analysis performed on the MCPyV positive samples showed some nucleotides differences with respect to the reference MCC350, strain EU375803 [62] that did not produce stop codons or any amino acid change in the derived protein sequence LT and VP1.

4.3 Microbiology in MCPyV positive and negative CF patients

Within the CF patients' respiratory samples (n = 1138), the most common bacteria detected were *S. aureus* (n = 397), *P. aeruginosa* (n = 268) or both bacteria (n = 227) (**Table 4**). The concomitant detection of MCPyV-DNA with *S. aureus* or *P. aeruginosa* was observed in 103/1138 samples (9.1%) and in 61/1138 samples (5.4%) respectively, whereas the co-detection of MCPyV DNA, *S. aureus* and *P. aeruginosa* was found in 48/1138 samples (4.2%). No significant association was found between MCPyV-DNA detection and the microbiological status of CF patients and also analyzing MCPyV positive individuals stratified by age (0–10 years, 11–24 years, ≥25 years; $p > 0.05$) (**Table 4**). Given that mucoid *P. aeruginosa* has been associated with more severe pulmonary disease [330], we examined the phenotype of *P. aeruginosa* according to the MCPyV positivity. We did not find any difference in the prevalence of the mucoid phenotype of *P. aeruginosa* between MCPyV positive (26.5%) and negative (24.8%) CF patients and also examining MCPyV positive patients stratified by age (0–10 years, 11–24 years, ≥25 years; $p > 0.05$) (**Table 4**).

4.4 Gene expression of TLR9 and IFN-I subtypes in MCPyV positive and negative CF patients

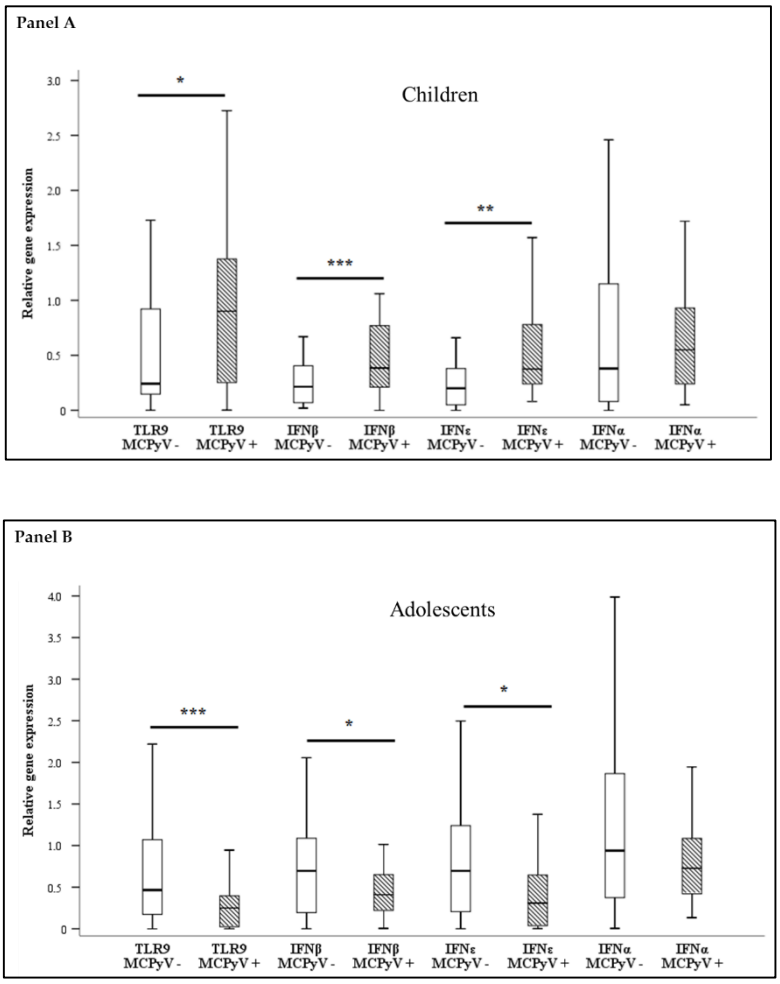
Considering that patients' age has an impact on the innate immune response to respiratory viruses [331,332], we compared TLR9 and IFN-I gene expression levels between positive and negative MCPyV patients stratified in age groups (0–10 years, 11–24 years, ≥25 years). Demographic and clinical characteristics of CF patients analyzed are reported in **Table 5**.

Table 5. Demographic and clinical characteristics of Merkel cell polyomavirus (MCPyV) positive (n=95) and negative (n=147) samples selected for TLR9 and IFN-I genes (IFNα, IFNβ and IFNε) analysis from the cystic fibrosis (CF) cohort patients.

Items	Total n=242	MCPyV + samples n= 95*	MCPyV - samples n=147*
Males, n (%)	122 (50.4)	44 (46.3)	78 (53.1)
Age, mean (SD)	26.4 (16.4)	25.1 (15.8)	27.3 (16.8)
ΔF508 homozygous/ heterozygous/others, n	110/58/62	43/24/25	67/34/37
BMI, mean (SD)	20.2 (3.7)	19.8 (3.5)	20.5 (3.9)
Exacerbation events, n (%)	20 (8.3)	10 (10.5)	10 (6.8)
Aspirate samples, n (%)	139 (57.4)	57 (60)	82 (55.8)
Sputum samples, n (%)	103 (42.6)	38 (40)	65 (44.2)
P. aeruginosa presence, n (%)	59 (24.4)	26 (27.4)	33 (22.5)
S. aureus presence, n (%)	77 (31.8)	29 (30.5)	48 (32.6)
P. aeruginosa and *S.aureus* presence, n (%)	54 (22.3)	17 (17.9)	37 (25.1)
Mixed microbial flora, n (%)	52 (21.5)	23 (24.2)	29 (19.8)
TLR9 (CV%)	0.27 (36.7)	0.23 (72.3)	0.31 (40.1)
IFNα (CV%)	0.92 (30.7)	0.72 (29.8)	0.98 (31.4)
IFNβ (CV%)	0.53 (37.1)	0.46 (46.6)	0.62 (37.9)
IFNε (CV%)	0.41 (48.8)	0.32 (45.0)	0.64 (52.3)

*Gene expression was performed in 95 MCPyV positive respiratory samples collected from 72 CF patients and 147 negative respiratory samples collected from115 patients. Data are presented as number or mean. N.A.: not applicable. Transcript levels of TLR9, IFNβ, IFNε and IFNα related to β-glucoronidase mRNA were calculated using 2^-ΔCt, and indicated as median. CV: coefficient of variation.

MCPyV positive children produced increased levels of TLR9, IFNβ, IFNε and IFNα genes compared to the negative ones ($p = 0.02$, $p < 0.001$, $p = 0.002$, $p = 0.27$) (**Figure 3, Panel A**), whereas both MCPyV positive adolescents and adults had lower mRNA levels of TLR9 and IFNs-I than the negative patients (adolescents: $p < 0.001$, $p = 0.01$, $p = 0.02$, $p = 0.26$; adults: $p = 0.04$, $p = 0.001$, $p < 0.001$, $p = 0.12$) (**Figure 3, Panel B and C**).



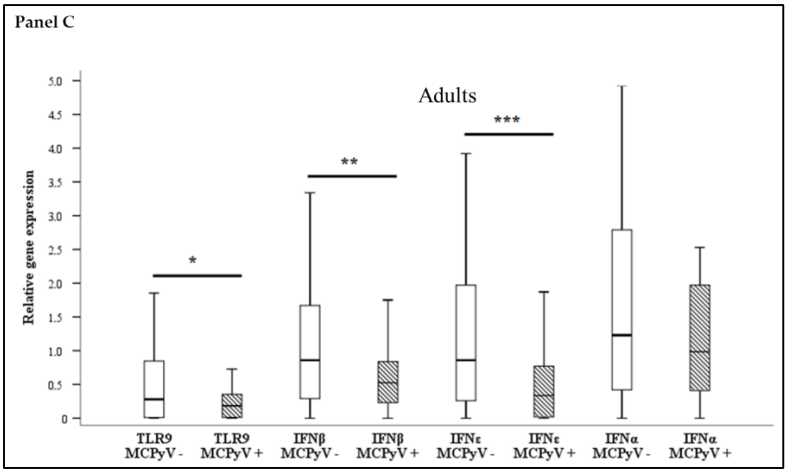
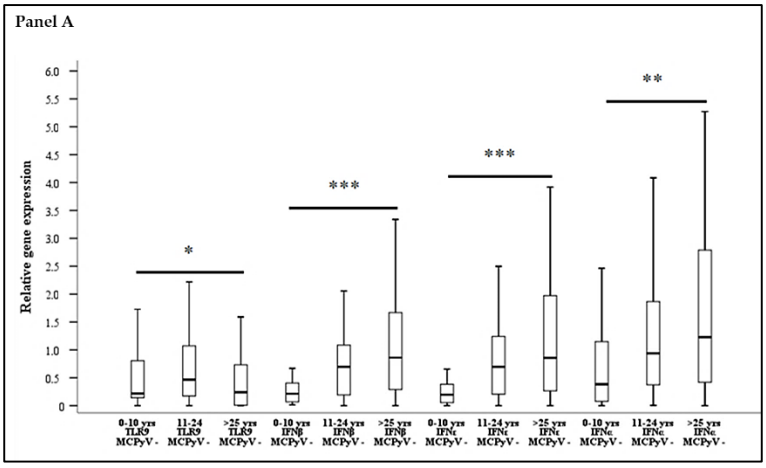
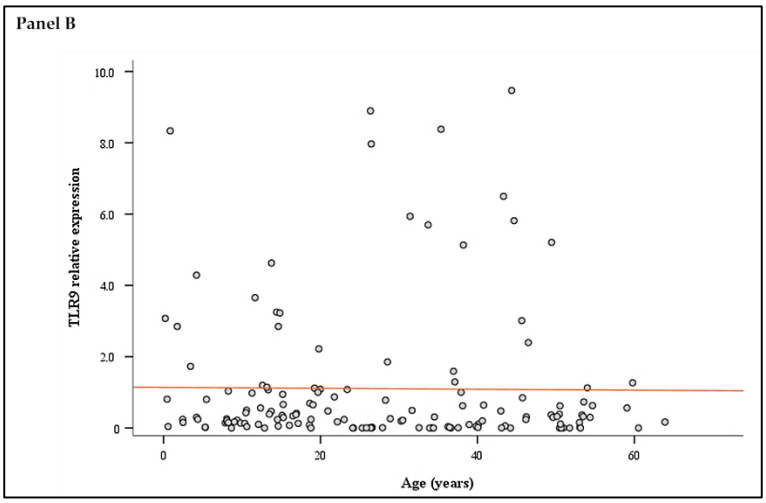
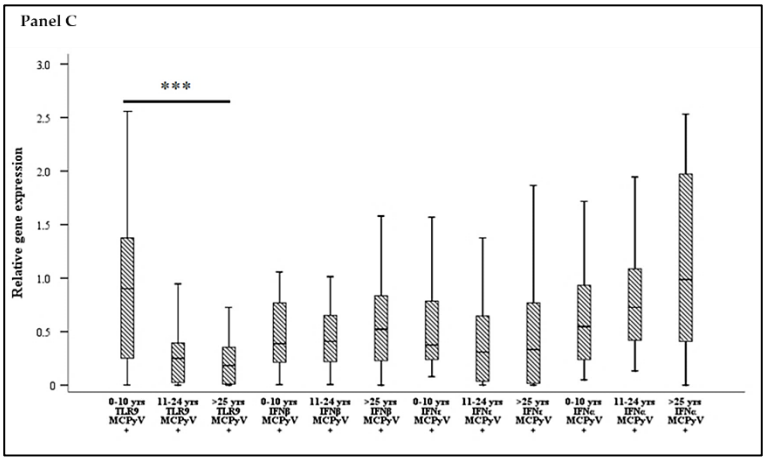


Figure 3. Expression levels of genes encoding TLR9, IFNβ, IFNε and IFNα in respiratory samples collected from cystic fibrosis (CF) patients stratified by age (0–10 years, 11–24 years, ≥25 years) without Merkel cell polyomavirus (MCPyV) (Panel A) or with MCPyV (Panel C). Scatter plot showed the correlation between age (years) and TLR9 mRNA levels expressed as relative expression in MCPyV negative patients (Panel B) and in MCPyV positive patients (Panel D). *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$.

Moreover, in the MCPyV negative group, IFNβ, IFNε and IFNα mRNA levels were lower in children compared to adolescents and adults ($p < 0.001$, $p < 0.001$, $p = 0.002$), while TLR9 was increased in adolescents and reduced in children and adults ($p = 0.03$, **Figure 4, Panel A**). On the other hand, analyzing the MCPyV positive group, we found that TLR9 levels were reduced in adolescents and adults compared to children ($p < 0.001$) while there were no significant differences in IFNβ, IFNε and IFNα mRNA levels between age groups ($p > 0.05$ for all genes, **Figure 4, Panel C**). In agreement with these results, transcript levels of TLR9 were found to be inversely correlated with age in MCPyV positive patients ($r = -0.34$, $p = 0.001$) (**Figure 4, Panel D**), differently from that observed in MCPyV negative patients ($r = -0.10$, $p = 0.22$) (**Figure 4, Panel B**).


Panel A
Relative gene expression
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
***
***
**
0-10 yrs TLR9 MCPyV -
11-24 TLR9 MCPyV -
>25 yrs TLR9 MCPyV -
0-10 yrs IFNβ MCPyV -
11-24 yrs IFNβ MCPyV -
>25 yrs IFNβ MCPyV -
0-10 yrs IFNτ MCPyV -
11-24 yrs IFNτ MCPyV -
>25 yrs IFNτ MCPyV -
0-10 yrs IFNc MCPyV -
11-24 yrs IFNc MCPyV -
>25 yrs IFNc MCPyV -


Panel B
TLR9 relative expression
10.0
8.0
6.0
4.0
2.0
0
0
20
40
60
Age (years)


Panel C
Relative gene expression
3.0
2.5
2.0
1.5
1.0
0.5
0
***
0-10 yrs TLR9 MCPyV +
11-24 yrs TLR9 MCPyV +
>25 yrs TLR9 MCPyV +
0-10 yrs IFNβ MCPyV +
11-24 yrs IFNβ MCPyV +
>25 yrs IFNβ MCPyV +
0-10 yrs IFNτ MCPyV +
11-24 yrs IFNτ MCPyV +
>25 yrs IFNτ MCPyV +
0-10 yrs IFNc MCPyV +
11-24 yrs IFNc MCPyV +
>25 yrs IFNc MCPyV +

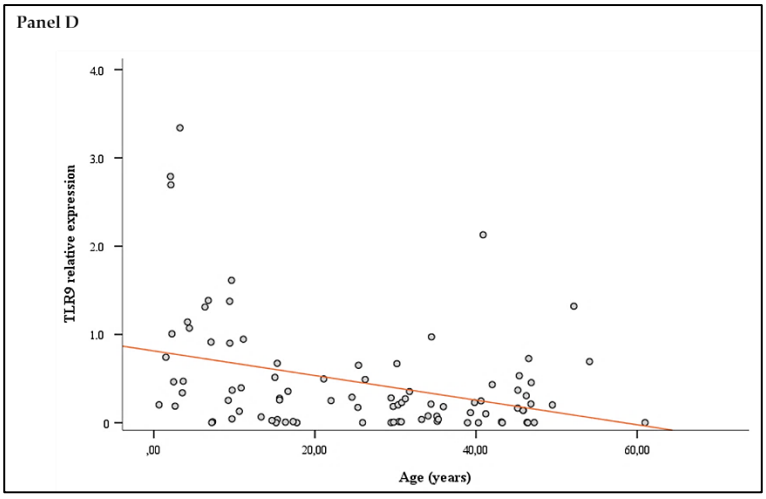


Figure 4. Expression levels of genes encoding TLR9, IFNβ, IFNε and IFNα in respiratory samples collected from cystic fibrosis (CF) patients stratified by age (0–10 years, 11–24 years, ≥25 years) without Merkel cell polyomavirus (MCPyV) (Panel A) or with MCPyV (Panel C). Scatter plot showed the correlation between age (years) and TLR9 mRNA levels expressed as relative expression in MCPyV negative patients (Panel B) and in MCPyV positive patients (Panel D). *p ≤ 0.05 **p ≤ 0.01 ***p ≤ 0.001.

4.5 Gene expression in MCPyV positive and negative CF patients according to the microbiological status

Given that bacterial colonization might differently activate TLRs and IFN response [327,333], we further explored whether transcript levels of TLR9, IFNβ, IFNε and IFNα in MCPyV positive CF patients could be influenced by the microbiology status. Thus, CF patients were stratified according to the detection of *S. aureus*, *P. aeruginosa* or both. We found that TLR9 levels decreased in MCPyV-DNA and *P. aeruginosa* positive CF patients compared to those negative for MCPyV-DNA detection (p = 0.009, **Figure 5**).

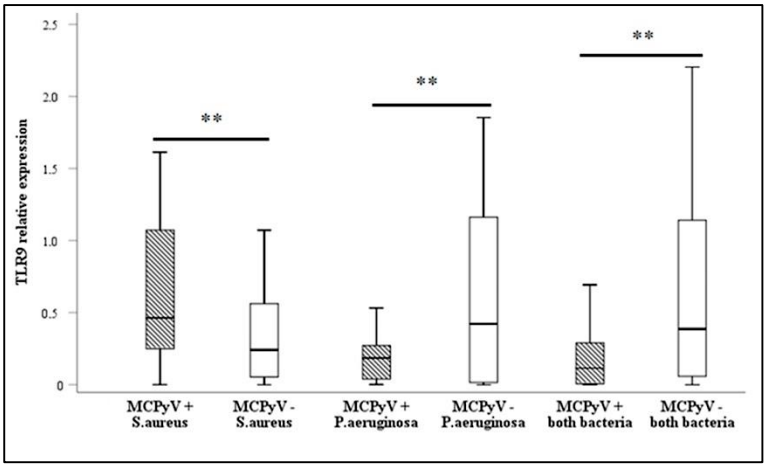


Figure 5. Expression levels of TLR9 mRNA in respiratory samples collected from cystic fibrosis (CF) patients with or without Merkel cell polyomavirus (MCPyV), stratified according to the bacteriological status: *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and both bacteria. **$p \leq 0.017$

By contrast, TLR9 levels were higher in *S. aureus* colonized MCPyV positive CF patients than in the negative ones ($p = 0.006$, **Figure 5**). Differently, when CF patients were infected by *P. aeruginosa* and *S. aureus*, TLR9 levels were higher in those negative for MCPyV-DNA than in those positive for MCPyV-DNA ($p = 0.003$, **Figure 4**). There were no differences in mRNA levels of IFNβ, IFNε and IFNα between MCPyV positive and negative CF patients suffering from *S. aureus*, *P. aeruginosa* or both bacterial infections ($p > 0.05$, data not shown).

4.6 Exacerbation events

Whether there was an association between the detection of MCPyV-DNA in respiratory specimens, the alteration in TRL9 and IFN-I levels and the frequency rates of acute exacerbations observed in CF patients was assessed. Exacerbation data were available for 1108/1138 respiratory samples (97.4%) collected from 516/539 CF patients (95.7%): 89 CF patients presented exacerbation. The number of CF individuals with respiratory exacerbation was similar between those positive (n = 27/ 266, 10.1%) or negative (n = 62/842, 7.4%) for MCPyV-DNA (**Table 6**).

Table 6. Demographic and microbiological characteristics of Merkel cell polyomavirus (MCPyV) positive (n=266) and negative (n=842) cystic fibrosis (CF) samples.

Items	MCPyV-DNA (+) samples (n=266)		MCPyV-DNA (-) samples (n=842)	
	Exacerbated (n=27)¤	Non exacerbated (n=239)	Exacerbated (n=62)¤	Non exacerbated (n=780)
0-10 years, n (%)	6 (22.2)	67 (28.0)	11 (17.7)	168 (21.5)
11-25 years, n (%)	4 (14.9)	71 (29.7)	12 (19.3)	248 (31.8)
>25 years, n (%)	17 (62.9)♦	101 (42.3)	39 (63.0)●	364 (46.7)
P. aeruginosa, n (%)	9 (33.3)	52 (21.7)	26 (41.9)	175 (22.5)
S. aureus, n (%)	10 (37.0)	93 (38.9)	12 (19.3)	274 (35.3)
P. aeruginosa and *S. aureus*, n (%)	6 (22.2)	41 (17.1)	15 (24.2)	161 (20.7)
Mixed microbial flora, n (%)	2 (7.4)	53 (22.2)	9 (14.5)	167 (21.5)

Data are presented as number. ¤Respiratory exacerbation rate was similar between those positive or negative for MCPyV (p=0.16 by Chi-square test); ●Exacerbation events were higher in the adult group compared to adolescents and children in MCPyV negative patients (p=0.04 by Chi-square test); ♦Exacerbation events did not differ according to the age of MCPyV positive patients (p=0.12 by by Chi-square test).

Exacerbation events were higher in the adults compared to adolescents and children in MCPyV negative patient's group (p = 0.04, **Table 4**), while they did not differ according to the age of MCPyV positive patients (p = 0.12, **Table 6**). Comparing exacerbated and non-exacerbated CF patients, we found a higher frequency of acute respiratory events in MCPyV negative patients with *P. aeruginosa* infection (p = 0.003, **Table 6**), while a similar frequency of exacerbation events was recorded in MCPyV positive individuals with *S. aureus*, *P. aeruginosa* or both bacteria (p = 0.20, **Table 6**). A logistic regression analysis was carried out to develop a model of risk factors for

respiratory exacerbation occurrence in MCPyV positive CF patients. The detection of *S. aureus* (OR 2.45, 95% CI 0.89–6.68; p = 0.08) or *P. aeruginosa* (OR 0.69, 95% CI 0.26–1.78; p = 0.446) was not associated with a higher frequency of pulmonary exacerbations episodes in MCPyV positive individuals. We also found no difference between exacerbated and non-exacerbated CF patients in both MCPyV positive and negative patients according to TLR9, IFNβ, IFNε and IFNα mRNA levels ($p > 0.05$, data not shown).

Objective two:

4.7 Detection of the MCPyV DNA by standard PCR and quantification of viral loads by Quantitative real-time PCR (qPCR)

FFPE from 112 NSCLCs and corresponding nonmalignant lung tissue, were screened for the presence of MCPyV DNA by standard PCR with three sets of primer, LT1, LT3, and VP1, commonly used to detect the MCPyV LT and VP1 genes [62]. Twenty out of 112 lung cancers (17.9%) harboring MCPyV DNA. In contrast, MCPyV DNA was detected in only one nonmalignant lung tissue (0.9%). Specifically, among the 112 NSCLC, of the 32 SCCs, MCPyV DNA was detected in 3 tumors with the LT1 primers, in 7 tumors with the LT3 primers, and 4 tumors with the VP1 primers (**Table 7**). Of the 45 ACs, MCPyV was found in 7 tumors with the LT1 primers, 8 tumors with the LT3 primers, and 5 tumors with the VP1 primers (Table 7). Of the 32 LCCs, MCPyV DNA was detected in only one sample with the LT3 primers (**Table 7**). In one of three patients with PL, the MCPyV DNA was detected with LT3 and VP1 primer sets (**Table 7**). Of these 20 samples, six tumors (SCC10, SCC27, AC7, AC35, AC38 and AC41) contained MCPyV DNA sequences amplifiable by all three primer sets (**Table 7**). The MCPyV-DNA-positive samples were subjected to qPCR. The viral DNA was detected in the same analyzed samples with a viral load that ranged from $1x10^2$ to $5,5x10^2$ copies per μg (**Table 7**). MCPyV DNA detected in the nonmalignant lung tissue shared a viral DNA load of $1,5x10^2$ copies per μg. No significant association was found between the presence of MCPyV and the other clinic-pathological features analyzed. However, MCPyV was more frequently detected in never smokers than in smokers, although this difference was not statistically significant.

Table 7. Evaluation of the MCPyV prevalence in NSCLCs and study of a possible viral etiology of NSCLCs by examining for both the presence of specific MCPyV DNA and the expression of viral RNA transcripts.

				PCR			Real Time PCR	RT-PCR	
Case	Age	Sex	Smoking Status	LT1	LT3	VP1	MCPyV DNA load (copies/ug)	LT-RNA	VP1-RNA
SCC1	71	F	N	+	-	-	1,00E+02	NT	
SCC10	76	M	F	+	+	+	1,75E+02	+	-
SCC12	73	M	F	-	-	+	4,00E+02	-	-
SCC20	70	M	F	-	+	-	2,50E+02	NT	
SCC22	72	M	N	-	+	-	5,50E+02	-	-
SCC24	71	M	N	-	+	+	2,25E+02	NT	
SCC25	70	M	F	-	+	-	3,75E+02	-	-
SCC27	74	F	N	+	+	+	1,25E+02	NT	
SCC31	81	M	N	-	+	-	4,50E+02	NT	
AC2	79	M	F	+	+	-	1,00E+02	NT	
AC3	60	M	F	+	-	+	1,25E+02	NT	
AC7	72	F	N	-	+	-	2,35E+02	-	-
AC14	73	F	N	+	+	+	5,00E+02	-	-
AC30	73	M	F	-	+	-	4,50E+02	NT	
AC32	72	F	F	+	+	+	2,50E+02	NT	
AC35	69	M	F	+	+	+	3,00E+02	+	-
AC38	65	F	F	+	+	+	3,25E+02	+	-
AC41	80	F	N	+	+	-	1,75E+02	+	-
LCC25	80	M	F	-	+	-	1,00E+02	NT	
PL1	70	M	F	-	+	+	2,00E+02	-	-

4.8 Expression of LT and VP1 transcripts

Specimens from 10 out of the 20 MCPyV-DNA-positive tumors were suitable for RNA extraction (SCC10, SCC27, AC2, AC3, AC7, AC14, AC32, AC35, AC38 and AC41). The expression of the MCPyV LT (nucleotide positions 910–1152, corresponding to exon 2) and VP1 (nucleotide positions 3786– 4137) transcripts was examined at the RNA level by RT–PCR. Four of ten samples expressed the LT gene transcript (**Figure 6**), whereas no VP1 gene transcript was found in any samples (**Figure 6**).

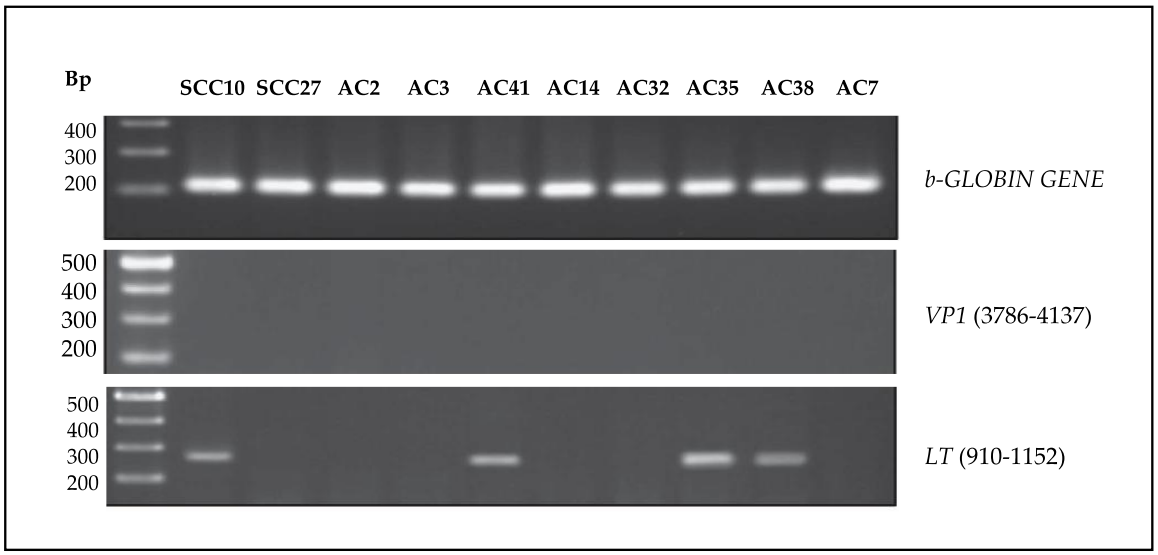


Figure 6. Expression of the LT and VP1 gene transcripts. RNAs were reverse transcribed and the cDNAs were PCR amplified with primers complementary to the regions corresponding to the LT or VP1 genes. All cDNAs were also subjected to amplification in parallel with the housekeeping gene b-globin, which was expressed at similar levels in all samples. Case numbers are indicated on the top. Molecular weight markers are shown on the left.

4.9 Integration of the MCPyV genome

The quantity and quality of the DNA are critical for the DIPS–PCR analysis, and the DNAs from 15 MCPyV-DNA-positive samples were suitable for this analysis. The integration site was identified in one sample (SCC10) in which, the virus–host junction was located at nucleotide position 2738 of the MCPyV LT gene with the viral DNA sequence was inserted into the long arm of chromosome 5 (5q23.1)

4.10 DNA sequencing analysis of the MCPyV LT gene

DNAs from four tumors, SCC10, AC35, AC38, and AC41, were subjected to a sequence analysis of the full-length LT gene at nucleotide positions 151–3102. According to GenBank data, the wild-type *non*-tumor-derived MCPyV strain, Appendix206 (JN038578), has a lysine at amino-acid position 216 within a penta-amino-acid retinoblastoma tumor-suppressor protein (Rb)-binding motif (LFCDK) encoded by exon 2 of the LT gene, whereas its substitution with a glutamate (LFCDE), is found in the MCC tumor-derived MCC350, EU375803 strain [62]. The

MCPyV strains found in the four patients were consistent with the MCC350, EU375803 strain [62] and contained the LxCxE motif (**Figure 7**). The psycho motif is also known to modulate Rb activities [334]. MCPyV has a unique psycho motif that is separated by a spacer region at amino-acid positions 83–102 and at amino-acid positions 210–218 [335]. Several amino-acid differences between Appendix206 and MCPyV analyzed strains were found in this spacer region. Of these, the MCPyV strains in our samples shared the amino-acid substitution at position 112 (H to Y), substitutions at positions 121 (S to P) and substitutions at positions 156 (P to S) with the MCC350, EU375803 strain [62]. The full-length LT gene sequence could be amplified and, although several non-synonymous mutations, resulting in amino-acid substitutions, were present at the C terminus of LT, no mutations, causing stop codons, were observed in samples (SCC15, AC35, AC38) (**Figure 7**). On the other hand, in sample AC41, a frameshift mutation from nucleotide positions 1611–1656, generated several stop codons. These mutations occurred downstream from the Rb-binding domain and caused a truncated exon 2, which encodes the LT helicase (**Figure 7**).

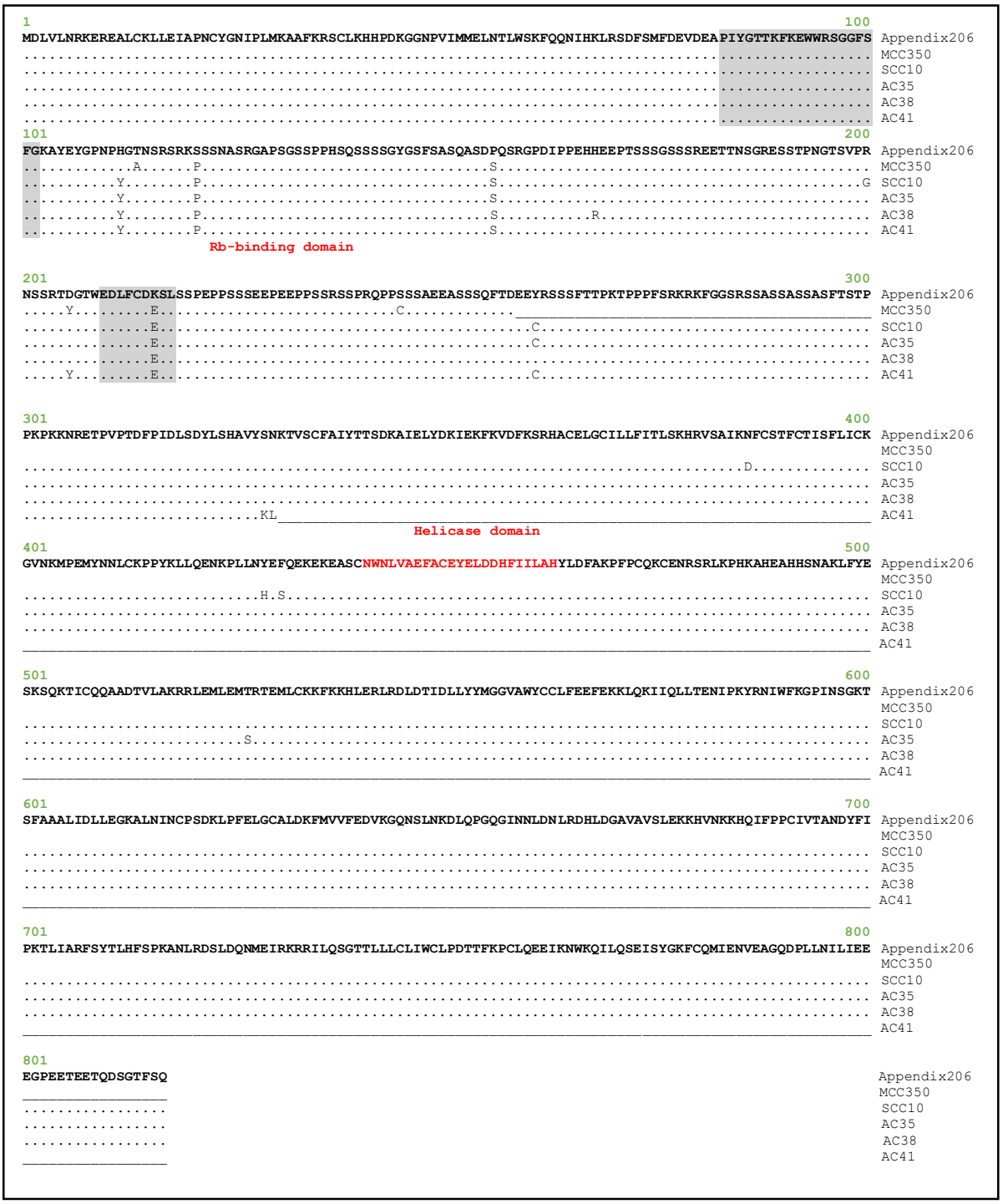


Figure 7. Amino-acid sequence alignment of the MCPyV LT antigen. The LT genes in the MCPyV-positive tumors seem to encode 817 amino-acid proteins. The amino-acid sequences were compared with reference sequences from the non-tumor-derived MCPyV isolate Appendix206 (GenBank accession number JN038578) and the MCC-tumor-derived isolate MCC350, strain EU375803 [62]. The position of the LxCxE motif, which is essential for Rb binding, is shown by the upper line. The psycho motif, which influences Rb activities, is represented with grey boxes. This motif, interrupted by amino-acids at positions 103–209, is shown in a white box with black lines. The arrows indicate the

position of the helicase domain. The bold lines indicate the truncated regions of the LT antigen found in MCC350, strain EU375803 [62]. The numbers indicate the amino-acid positions.

4.11 EGFR mutations analysis

EGFR mutations were analyzed in 112 NSCLCs and in corresponding nonmalignant lung tissue. Results showed that mutations were found in 27 of 112 NSCLCs patients (24%) and only occasionally in the corresponding nonmalignant lung tissue. Specifically, the EGFR gene displayed mutations in exons 19-21. Deletion of exon 19 and the substation L858R in exon 21 are two main mutation types observed. Mutations were more common in women (P = 0.009) and never in smokers (P= 0.0001). Studying the infection rate of MCPyV, it was observed that this was higher in NSCLCs with EGFR mutations (30.2%) than without EGFR mutations (15.8%); however, this difference was not statistically significant (P= 0.061).

5. DISCUSSION

Although MCPyV is known as a critical factor in the development of MCC [62], MCPyV-DNA has been detected in a variety of *non*-MCC cancers, including chronic lymphocytic leukemia, malignant tonsillar tissues, cervical carcinomas, non-melanoma skin cancers, and lung cancer [336] and recently in diverse specimens, including respiratory tract samples [295,337-340]. As hypothesized for other HPyVs [341-342], MCPyV could be transmitted by inhalation through the respiratory route and then spread to other sites, such as epidermal tissue. The role of MCPyV as a respiratory pathogen remains highly speculative since patients generally remain asymptomatic or exhibit nonspecific upper respiratory tract symptoms. Beside the role as respiratory pathogen, MCPyV infection in the respiratory tract mucosal cells may cause a dysregulation in the innate immune sensing and response to other microorganisms.

The impact of respiratory viruses has been only recently recognized in CF patients [343]. Increased respiratory symptoms, decline of respiratory function, higher frequency and duration of hospitalization have been reported in CF for HRV, RSV and Influenza A/B viruses [343]. However, microbiological and clinical implication of respiratory viruses in CF remains poorly understood.

In this study, the prevalence of MCPyV-DNA in respiratory samples, collected from CF patients, reached 23.5%, a rate higher than those reported in previous studies in *non*-CF individuals [295,307,337-339], validating the hypothesis that microenvironment conditions in CF respiratory tract could promote a higher rate of MCPyV infection and/or the frequency of its reactivation from latency [311,313]. We did not observe a statistically significant difference in MCPyV prevalence and viral load stratifying CF patients according to age and gender or microbiology status.

These data confirm what is already hypothesized for CF patients: the rate of positivity for respiratory viruses is similar among CF children and adults [344],

although data about viral prevalence remain highly under-reported, especially in the adults, because, not all CF patients, have severe symptoms during viral infections and also for technical difficulties as the use of incomplete PCR panels to routinely detect the respiratory viruses [345]. Adults with CF have a lower prevalence of respiratory viral infections associated with pulmonary exacerbations compared to children and infants [346]. Also, there was no clear seasonal trend for MCPyV-DNA detection in respiratory samples from CF patients. However, in agreement with literature [311,313], a higher frequency of MCPyV positivity was reported in February, October and December suggesting that, cold weather and low relative humidity, could promote MCPyV replication and spreading. Moreover, our data showed that MCPyV could persist for extended periods in the respiratory tract of CF patients: thirteen patients remained MCPyV-DNA positive in four consecutive respiratory samples for a median follow-up period of about 9 months. Although HPyV reactivation in the respiratory tract was observed in hematology/oncology and AIDS patients [347], this result suggests that CF patients might have a greater risk of MCPyV reactivation, perhaps because of a persistent microbial stimulus in their respiratory tract [311].

In order to improve the knowledge of NCCR alterations in MCPyV strains circulating in CF, the NCCR variability was also analyzed. It has been established that modifications in NCCR structure are the main event in the onset of HPyVs-related pathology, as demonstrated for other HPyVs such as JCPyV and BKPyV, in which NCCRs control gene expression and, harboring the origin of DNA replication and transcription factor binding sites, represent a key determinant in viral replication [347]. On the other hand, relatively little is known about the role that NCCR plays in MCPyV infection, and limited data are available on the relationship between MCPyV NCCR variability and pathogenesis. NCCR sequence analysis revealed a high degree of homology with the prototype MCC350, strain EU375803 [62] although transitions, transversions, single or double deletions and insertions were randomly observed. Differently to JCPyV and BKPyV, in which the upstream

or 5'side of NCCR is highly conserved and the downstream or 3'side undergoes rearrangements [348], we found that mutations occurred both in early and in late proximal side of the MCPyV NCCR.

Rearrangements in MCPyV NCCR have been associated with genotypes that vary with ethnicity [326]. Specifically, two major subtypes, I and II, were identified based on the presence or absence of a 25 bp tandem repeat into nucleotide positions 5177–5178 of the MCPyV NCCR. Based on the occurrence of two additional insertions (2 bp, TT, and 5 bp insertions, GTTGA, between nucleotide positions 5199–5200 and 5210–5211, respectively), MCPyV strains have been further assigned to five genotypes. In our analyzed strains, we found the MCPyV NCCR IIa-2 strain, which contains the 5 bp insertion GTTGA (nucleotide positions 5210–5211) and represents the predominant strain among white persons of European descent, as expected for our cohort of CF patients [326].

In this study, it was evaluated whether the nucleotide changes observed in MCPyV NCCRs involved the structure of putative binding sites for different cellular transcription factors [326,349-351]. Sequence analysis showed that the NCCR sequence (MCC350, strain EU375803 [62]) contains multiple binding sites such as NHP-1, FOXN2, BTEB3, FOXO3A, NF-AT3, FOXJ2, GATA1, AP1 and PAX2, already described within the NCCRs of other HPyVs [326,349-351]. In several strains obtained from MCPyV-positive samples, mutations fell within these putative binding sites, changing some of these motifs in RELA, STAT4, ETS-1 AR, HLF and Sp3.

The relevance of different cellular factors, including Tst-1, NF-1, Sp1, NF-kB and PURα, that specifically determine JCPyV tropism for glial cells and play an important role in favoring efficient HPyVs DNA replication, has been described [352]. Moreover, a potential association, between a C/G mutation in the NCCR Sp1 site and increased BKPyV virulence in hemorrhagic cystitis patients, has been proposed [353]. Considering these evidences, further studies are warranted in order

to define the importance of NCCR binding sites and understand how these changes may drive MCPyV replication and pathogenicity in vitro and in vivo.

In the attempt to understand whether changes in NCCR sequence could be correlated with an increased MCPyV replicative capacity, the viral load was analyzed considering the occurrence of these mutations. In CF patients who had positive results for MCPyV-DNA detection in four consecutive respiratory samples and in which no mutations were observed, the number of MCPyV-DNA copies/mL decreased over the time. Conversely, a higher number of MCPyV-DNA copies/mL was reported in CF patients with GTTGA insertion. As previously reported for BKPyV and JCPyV, rr-NCCRs conferred a higher replication rate to these viruses, contributing to disease progression [354]. Consequently, also for MCPyV, it is possible to speculate that the shift from canonical NCCR to rr-NCCR could determine higher replication capacity.

Since it is also likely that high MCPyV-DNA levels might enhance the chance of LT mutations or viral integration into the host cell genome and then, the oncogenic properties of MCPyV in a context different from that of MCC, the analysis of the LT sequence, was investigated in CF patients positive to MCPyV DNA.

Results showed that, although mutations were detected throughout the LT sequences, included at the C terminus of LT that contains anti-tumorigenic properties and may explain why this region is deleted in MCC [355], no mutations, causing stop codons and encoding a truncated protein, were detected.

In addition to LT truncation, also mutations in VP1 have been proposed to be indirectly involved in viral DNA tumorigenesis [356-358].

The overall analysis of VP1 sequences recovered in this study, showed a high degree of identity respect to the prototype MCC350, strain EU375803 [62]. Although some variations were observed throughout the VP1 sequences, none of these produced any amino-acid change in the derived protein.

Therefore, considering these results, it is possible to conclude that the oncogenic mechanism of MCPyV involving tumor-specific signatures of MCPyV in MCC was not observed in the context of CF.

Viral infections in combination with colonizing poly-microbial communities may differentially contribute to alteration in the airway inflammation and innate immune response in CF [359]. TLRs, involved in viral detection and microbial recognition, play a key role in regulating the inflammatory response in respiratory epithelial cells of healthy individuals and in those with chronic respiratory diseases, including CF individuals [327]. Hence, the presence of an excessive or reduced TLR expression and activation can interfere with inflammatory response [360]. Because the respiratory tract in CF is most frequently colonized by *S. aureus* and *P. aeruginosa,* that have been both involved in causing alterations in TLR and IFN pathways [361-363], we considered their presence as a relevant factor to understand if MCPyV could have a role in influencing IFN response.

Remarkably, the results presented here indicate that detection of MCPyV-DNA in respiratory samples of CF patients is associated with alteration in the transcript expression of TLR9. Surprisingly, MCPyV positive children had higher transcript levels of TLR9 and IFNs compared to the negative ones, while a reduction in TLR9 and IFNs mRNAs was found in adolescents and adults with MCPyV. The increased TLR9 and IFNs levels found in children with MCPyV might be caused by the activation of innate immune response as response to the first MCPyV infection occurring at the early ages; on the other hand, CF adolescents and adults might probably experience different MCPyV reactivations, resulting in abundant production of viral early genes able to modulate the innate immune responses [364]. Thus, MCPyV-mediated alteration of TLR9-mRNA production could confer a benefit against the intrinsic dysregulated inflammatory response observed during CF, in which the dysfunctional CFTR is known to trigger high proinflammatory cytokine levels either at basal or as consequence to viral or microbial infections [365].

Moreover, the finding that TLR9 levels were higher in MCPyV positive children, compared to those observed in MCPyV positive adolescents and adults, could be partially explained by a more frequent detection of *S. aureus* in children: in this regard, *in vitro* studies have identified TLR9 as one of the most important pattern recognition receptor involved in the induction of IFN-I signaling in response to *S. aureus* infection [366,367]. However, on one hand TLR9 was found to be inversely correlated with age in MCPyV positive CF patients, on the other hand TLR9 was higher in MCPyV negative adolescents, suggesting the existence of a potential combined effect of MCPyV and *S. aureus* co-detection on TLR9 expression in children. Indeed, CF adolescents presented *S. aureus* as the most frequent bacterium in their respiratory tract, highlighting the complexity of the phenomenon analyzed. This hypothesis seems to be supported by the fact that TLR9 levels were enhanced in *S. aureus* colonized MCPyV positive CF patients than in the negative ones, while TLR9 levels decreased in those with *P. aeruginosa* and MCPyV compared to those negative for MCPyV-DNA detection. Indeed, the role of TLR9 played in response to *P. aeruginosa* remains not yet characterized thoroughly, although its contribution to the inflammatory response, up-regulation of TLRs, and bacterial clearance has been reported [368]. However, neutrophils appear to be capable of responding to *P. aeruginosa* DNA in a TLR9-independent manner [369], highlighting a complex interplay between bacteria, virus and innate immunity.

In this study, results point out that the detection of MCPyV-DNA in CF respiratory samples did not affect the frequency of pulmonary exacerbation events. In relation to the microbiology status of CF patients analyzed, exacerbation events in MCPyV negative patients were more frequent in individuals with *P. aeruginosa* infection, while a trend toward a major number of acute respiratory events was found in those positive for both *S. aureus* and MCPyV. In agreement with this data, while acute respiratory events in MCPyV negative patients were more frequent in adults, who usually present *P. aeruginosa* colonization [370], we did not find a significant

difference in the frequency of exacerbation events among the age groups in MCPyV positive patients.

In conclusion, MCPyV-DNA is frequently detected in the respiratory samples of CF patients, with a higher prevalence during cold months. Moreover, these results suggest that MCPyV could be involved in the reduction of TLR9 and IFNs transcript levels in both adolescents and adults with CF. By contrast, the detection of MCPyV-DNA and *S. aureus* is associated to an increase of TLR9 and IFNs expression levels in respiratory samples collected from CF children.

Overall, these observations indicated that MCPyV might differentially alter the expression of TLR9-mRNA in the respiratory tract of CF patients according to their bacteriological status, affecting indirectly the IFN-I production.

Recently, the seroprevalence and the possible co-infection of KI and WU polyomavirus (KIPyV and WUPyV, respectively) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), in respiratory samples of patients affected by coronavirus disease 19 (COVID-19), were exanimated [371,372]. Results confirmed that respiratory HPyVs were detected, although at low prevalence, also in symptomatic COVID-19 patients and that KIPyV and WUPyV could potentially drive viral interference or influence disease outcomes for example, in the context of COVID-19, by upregulating SARS-CoV-2 replicative potential [371]. Because analogous to KIPyV and WUPyV, also MCPyV has been observed in respiratory samples from patients affected by different form of disease, including CF patients, further studies are required to better evaluate whether these viruses can promote changes in microbial environment and mucosal immunological response.

Based on the histological similarities between MCC and pulmonary neuroendocrine carcinoma (NEC) of the skin, including SCLC and large-cell NEC (LCNEC), the possible association between MCPyV and pulmonary NEC, has been studied [243]. On the contrary, the prevalence of MCPyV in NSCLCs has not been investigated well and, for the best of our knowledge, the prevalence of MCPyV in NSCLC in an Italian cohort of NSCLC patients, has not yet been reported.

For this reason, as second aim of the study, the MCPyV prevalence and the possible viral etiology in NSCLCs were investigated by examining for both the presence of specific MCPyV DNA and the expression of viral RNA transcripts.

Our results provide the first evidence of the prevalence of MCPyV in Italian NSCLCs patients, in which the MCPyV infection rate was of 17.9%.

Many causative factors for lung cancer have been identified, including occupational agents, radiation, environmental pollutants, and smoking, the latter considered the major risk factor. As expected, most patients in our cohort were smokers (71.42 %), but one fourth of the patients (28.6 %) were never smokers. Specifically, 22 out of 32 of women and 5 out of 80 of men with lung cancer, were never smokers. Our results show a trend in which, a higher detection rate of MCPyV was observed in never smokers than in smokers. Thus, smoking behavior can not completely explain the characteristics of lung cancer and further studies are required to confirm this result.

In addition to causative factors listed above, also genetic, hormonal factors and infections may play a role in the development of lung cancer. To define MCPyV as *co-causative* infectious agent associated with lung cancer, MCPyV DNA positivity alone is not sufficient to establish this etiological role since, as reported also in the context of CF, MCPyV DNA sequences were detected in the respiratory tract when a high-sensitive PCR assay was used without tumor-specific signatures of MCPyV [313,373,374].

Three sets of primers, LT1, LT3, and VP1, frequently employed to detect MCPyV by PCR, were used also in this study and, in concordance with previous reports, the LT3 primers showed the highest sensitivity [357]. Several studies have demonstrated that the expression of the MCPyV LT antigen is a pivotal event for the oncogenesis of MCPyV-positive MCC [355]. In this regard, the expression of the LT gene and protein, at the RNA levels, were investigated. We found that 4 out of 10 MCPyV-DNA-positive NSCLCs had detectable levels of LT gene transcripts, whereas VP1 gene transcripts, in all the analyzed samples, were undetectable. This result could be explained assuming that, like other HPyVs also MCPyV displays, during viral replication, a gene expression that occurs in a specific sequence: the LT gene transcript is expressed first followed by the expression of the VP1 gene, because of the transcription of late viral genes [355,375]. The loss of the viral replication capacity is a common characteristic of virus-associated tumors [376]. In most MCPyV-positive MCCs, viral replication could be hampered explaining why the LT gene and not the VP1 gene is constitutively expressed [62]. Based on this fact, it is possible conclude that, the MCPyV strains isolated in this study, expressing the LT gene transcripts without VP1 gene transcripts, may not exhibit viral replication activity.

Another oncogenic mechanism of MCPyV involves the capacity of the LT antigen to bind the tumor-suppressor protein Rb by the conserved LxCxE motif [377]. Therefore, MCPyV presenting a conserved Rb-binding domain in the LT gene, may have a role in the tumorigenesis mechanism. In the MCPyV strains analyzed in this study, the LxCxE motif not only was preserved but also contained a conserved psycho motif, which modulates Rb activities [334]. Since the psycho motif of MCPyV shared limited homology to those of other HPyVs LT antigens and is interrupted by several amino acids [335], the roles of these substitutions remain to be clarified.

The integration of MCPyV into the host cellular genome is also considered as a key element in oncogenesis and, for this reason, the MCPyV status, using the DIPS–PCR method, was also investigated. The MCPyV integration site was identified in only

one sample (SCC10) in which, the virus–host junction was in the MCPyV LT gene at nucleotide position 2738 and the viral DNA sequence was inserted into the long arm of chromosome 5 (5q23.1). Although, to date, the integration sites identified in MCPyV-positive MCCs differ from one another, study reported that chromosome 5 is the preferred integration site [243,378]. The integration site found in sample SCC10 was localized within exon 2 of the LT gene, downstream from the Rb-binding domain. This result is congruent with the results of previous studies, in which most MCPyV-positive MCCs harbored the integrated viral genome at the end of the LT gene [243,272,379]. It is important to note that, the full-length LT gene sequence showed a non-truncated protein. This finding could suggest the coexistence of an integrated and an episomal forms of MCPyV. This phenomenon has been observed in MCPyV-positive MCCs [243]. Sequencing of full-length LT gene of the remaining three samples (AC35, AC38 and AC41), that did not show integration, displayed frameshift mutations that, although preserved the Rb-binding domain, truncated in one case (AC41) the oncoprotein before the helicase domain, as observed in most MCPyV-positive MCCs as tumor-specific molecular signatures [5]. Our RT–PCR data, showed the expression of the LT gene and the absence of VP1 gene expression. Truncations can avoid the transformation capacity of LT by the Rb sequestration eliminating viral DNA replication.

Taken together, the results obtained in this section provide evidence of the detection of MCPyV DNA and the expressions of both LT RNA transcripts in NSCLCs. Furthermore, it was demonstrated the presence of the integrated and mutate forms of MCPyV in a specific cancer *non*-MCC.

Mutations within LT and viral integration are considered the hallmarks of MCPyV-positive MCCs and a prerequisite for tumorigenesis.

Although the prevalence of MCPyV and the viral loads detected in Italian NSCLCs patients were low compared with those in MCPyV-positive MCCs, our data suggest a causative, rather than casual role of MCPyV in the pathogenesis of NSCLC.

The last although not the least analysis carried out in this study concerned the association between MCPyV infections and EGFR mutations was analyzed. Xu *et al.* [302] reported an association between MCPyV and EGFR mutation-driven NSCLC and suggested the possible role of MCPyV in the development of NSCLC. Studies have reported that EGFR mutations in NSCLC are associated with poor survival, frequent lymph node metastasis, and reduced chemo-sensitivity [380]. Thus, we studied the association between MCPyV infections and EGFR mutations, in MCPyV-infected NSCLCs. Results showed that the MCPyV rate was higher in NSCLC patients with EGFR mutations (29.8 %) compared to those without EGFR mutations (15.26 %). Although this difference was not statistically significant, probably due to the small number of EGFR-positive NSCLC patients, could suggests a possible role for MCPyV in inducing EGFR mutations in lung tissue. Moreover, since it is well-known that different EGFR mutations in EGFR exons 18,19,20 and 21 are related to different tyrosine kinase inhibitors (TKIs) sensitivity [302], study the EGFR role in lung cancer, could help to clarify if TKI could become the best choice for the treatment of patients with lung cancer.

In summary, our investigations underline that MCPyV-DNA is frequently detected in the respiratory samples of CF patients and that it might influence the expression levels of IFN-related genes in an age dependent manner. The factors that contribute to the infection and persistence of MCPyV in the airways must be well defined. It is possible to assume that MCPyV infection is one amongst other risk factors, leading to a malignant transformation of Merkel cells in human. It is possible to speculate that the continuous exposure of the lung tissues to MCPyV may cause viral integration and/or the expression of the mutated LT sequence, thereby leading to the oncogenic transformation of the infected host cells via MCPyV-associated tumorigenic pathways. Alternatively, viral integration itself might indirectly promote additional cellular changes during the carcinogenesis process, which allow the outgrowth of the tumor. The novel findings described in this study, should

stimulate further worldwide epidemiological and virological studies to determine unequivocally the pathogenetic relevance of MCPyV in lung tissue.

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