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Merkel cell polyomavirus, a small DNA tumour virus, in humans

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1. Introduction

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1.1 Polyomaviruses

Polyomaviruses (PyVs) are small, non-enveloped, double-stranded DNA viruses which infect a variety of hosts, from mammals to birds. PyVs are typically highly species-specific as they only infect the species from which they have been isolated. The first isolated PyV was the murine polyomavirus (MPyV). It was identified in 1958 following the observations of filterable extracts from murine leukaemia, which induced tumours in the parotid gland of newborn mice (1). An additional PyV, named Simian Virus 40 (SV40), has been isolated from African green monkey kidney cells, which were used during the production of polio vaccine, till 1963 (2,3). These two PyVs, MPyV and SV40, served as excellent models for studying the replication strategies of PyVs and how this family of viruses causes diseases, including tumours (1,2). In the past, PyVs were classified together with papillomaviruses in the family of Papovaviruses. However, in the 7th report of the International Committee on Taxonomy of Viruses, published in 2001, PyVs were reclassified into their own family, i.e., *Polyomaviridae* (4).

Polyomaviridae family is divided into three different genera: Orthopolyomavirus and Wukipolyomavirus, representing mammalian virus species, and Avipolyomavirus for avian virus species (Figure 1) (4). The most obvious difference between the genera is the host and cell specificity.

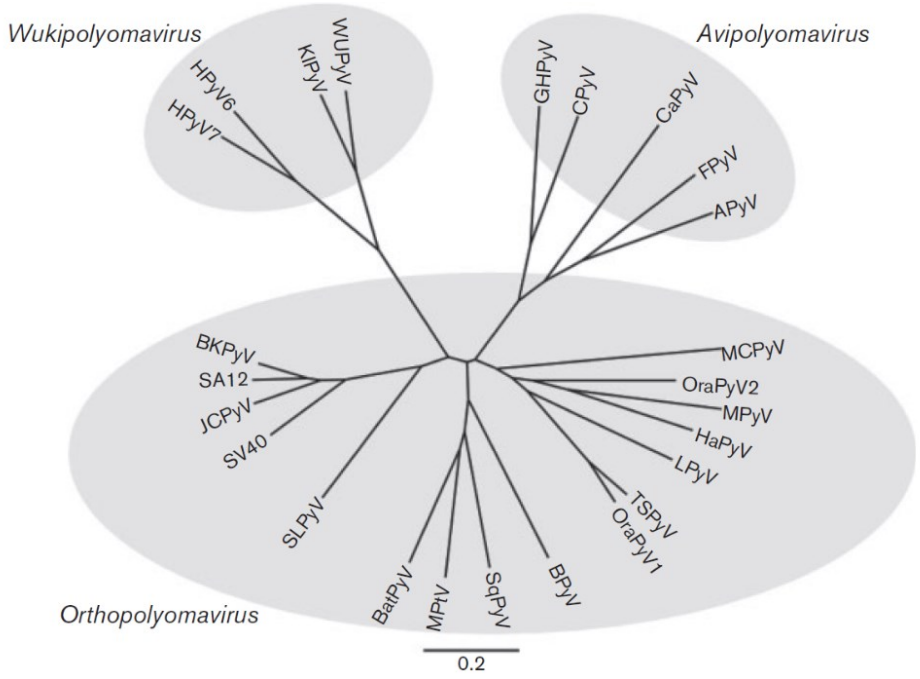


Figure 1. Phylogenetic tree of polyomaviruses based on whole genomic nucleotide sequence Adapted from Johne et al., 2011(5).

To date, fourteen humans PyVs (HPyVs) have been identified, thirteen since 2007. This increase in the viral identification is largely due to improved virus-detection technologies. One of the first identified HPyV was the John Cunningham PyVs (JCPyV). It was firstly isolated from the brain of a patient with progressive multifocal leukoencephalopathy (PML) in 1971 (6). JCPyV is a common virus that can infect neurons (7,8). About 50-90% of adults are infected with JCPyV, with a quarter of this subpopulation excreting JCPyV in their urine (8). As being an ubiquitous PyV, JCPyV does not exhibit exclusive tropism for a single cell type in its host, unlike other viruses. It is mainly found in oligodendrocytes, but also in tissues of the urinary tract, spleen, bone marrow (9), and even in chorionic villi and sperm samples (10,11). JCPyV infects 35-70% of the world population asymptomatically (12,13). The virus is found primarily in urban sewage systems, suggesting that it is ingested and spread via the faecal-oral route (14). The first site of JCPyV infection are tonsils, while it spreads and replicates in the lymphoid cells, followed by infection of the gastrointestinal tract and kidneys, where it causes latent infection (15). JCPyV has also been demonstrated to cause transformation *in vitro* of human and rodent cells and tumours in animal models. (4).

An additional important HPyV is BK (BKPyV) (10). It was firstly identified in 1971 by electron microscopic examination of urine from a renal allograft patient (16). Although the BKPyV route of transmission being not clear, BKPyV-infected healthy, and sick individuals transiently excrete viral particles that can be detected in various body fluids, such as urine, blood, and faeces. However, the most plausible route of infection is thought to be the respiratory tract (17). As a result of the increase in organ transplantation and the use of highly potent immunosuppressive drugs such as Mycophenolate mofetil (MMF) and tacrolimus in the early 2000, the reported incidence of BKPyV-related diseases is rising. Almost 10% of all kidney transplants show symptoms of BK-associated nephropathy (BKVAN), with approximately 50-90% of affected patients eventually experiencing graft dysfunction or failure (18).

Since the initial identification of JCPyV and BKPyV, numerous other human PyVs have been discovered, including the Washington University polyomavirus (WUPyV) and the Karolinska Institute polyomavirus (KIPyV) in 2007 (19,20). Both viruses have been firstly isolated from respiratory secretions, especially in children with severe pulmonary symptoms, but their role in causing pneumonia is not clear (19,20).

Thanks to the advantages of detection technologies such as next generation sequencing, more PyVs have been discovered in recent years. The latest HPyV was identified by Ondov and co-workers in 2019 (21). The sequences of the new HPyV, named

Quebec polyomavirus (QPyV), were detected in the stool of a patient using the MinHash algorithm (21,22).

The study of HPyVs and their link to human pathologies has becoming a more often important area of research. Notably, several proteins encoded by HPyVs have been associated with transforming abilities in cell cultures and animal models, thus suggesting a possible oncogenic role for these viruses. Other important diseases associated with these viruses include kidney infections and trichodysplasia spinulosa (4).

1.2 Merkel Cell Polyomavirus

Merkel cell polyomavirus (MCPyV, also known as human polyomavirus 5 [HPyV5]) is a naked, icosahedral, double-stranded DNA virus (23). MCPyV belongs to the *Polyomaviridae* family, while being the most recently discovered human oncogenic virus (4,24). Phylogenetically, it is quite distant from other known HPyVs and SV40, but more closely related to viruses of the chimpanzee and gorilla polyomavirus subgroups (25,26). Growing evidence suggests that MCPyV plays a causative role in the development of a highly lethal form of malignancy of the skin, named Merkel cell carcinoma (MCC) (24,27). Indeed, while the aetiological role of other PyVs, such as BKPyV and SV40 in human cancer remains controversial (28,29), MCPyV is broadly considered the main cause of a cancer in humans (24,27). Indeed, the World Health Organisation and the International Agency for Research on Cancer have classified MCPyV as a Group 2A carcinogen because there are strong evidences of a causal relationship between its presence and the occurrence of cancer in humans (30). Soon after primary infection, MCPyV evokes a physiological immune response, then it is maintained in a latent/persistent state lifelong in immunocompetent hosts (31). Impairments of the host anti-viral immune response can promote an increase in MCPyV replication activity, which ultimately leads to MCC tumorigenesis (31).

The MCPyV genome is a circular molecule of 5,387 nucleotides and comprises three functional domains, namely early and late coding regions and the non-coding control region (NCCR), located in between (Figure 2A) (32). NCCR comprises the viral origin of replication as well as promoters and enhancers that control the expression of the early and late viral genes (32). The late region encodes for genes expressed after the viral genome replication initiation as well as for two structural proteins named viral capsid protein 1 (VP1) and viral capsid protein 2 (VP2) and a viral microRNA (miRNA) (33–35). MCPyV genome is encapsidated in an icosahedral viral capsid consisting of VP1 and VP2. In particular, MCPyV virion consists of 72 pentamers of VP1, and each pentamer is lined by one molecule

of VP2 on the inner surface (36). Furthermore, VP1 plays a key role in the entry of the virus into the host cell. Indeed, VP1 mediates the initial binding of MCPyV on its host cell by binding sulphated glycosaminoglycans, specifically heparan sulphate proteoglycans (37).

Several transcripts are generated from the early region by alternative splicing (Figure 2B), including large T antigen (LT), small T antigen (sT), 57 kT antigen (57 kT) which is a spliced form of LT, and the alternative frame of the large T open reading frame (ALTO) (38). Due to alternative splicing events, LT, sT and 57 kT proteins share 78 amino acid (a.a.) residues in their N-terminal (N-Ter) region (Figure 2B) (39). The current knowledge on 57kT is remarkably poor. 57kT does not seem to support MCPyV replication, and its function in MCC carcinogenesis or infection is unclear (40). Since it shares the MCPyV unique region (MUR) with LT, while the helicase and origin binding domains are absent, it is hypothesised that 57kT indirectly supports MCPyV infection without directly supporting viral genome replication like LT. ALTO is encoded in an alternative open reading frame of LT (41). *In vitro* evidences obtained in HEK293 cells demonstrated that it is expressed during the MCPyV genome replication (41). However, ALTO does not seems to directly contribute to the viral genome replication in this cell culture but most likely may play an additional role in the viral life cycle (41).

MCPyV LT and sT present oncogenic potential in humans. LT contains several domains involved in viral genome replication and transcription. In particular, half of the LT N-Ter contains the DnaJ domain, followed by the HPDKGG motif responsible for binding to Hsc70 (33) and the MUR, which contains the conserved LXCXE retinoblastoma protein (RB) binding motif (42). Furthermore, a nuclear localization signal (NLS) containing RKRK sequence is located in the LT N-Ter (42). The remaining LT C-terminal (C-Ter) region includes an origin binding domain and helicase/ATPase domain, which are both needed for viral genome replication (Figure 2C)(43). The MCPyV sT antigen contains 186 a.a. residues, while sharing the 80 a.a. residues of the N-Ter with LT (38). The sT antigen comprises the DnaJ domain, but the RB motif is absent. The sT C-Ter region is unique while including two zinc-binding domains (CXCXXC motif) that provide structural and functional stability, and two domains rich in cysteine and proline residues that are responsible for the interaction of sT with protein phosphatase 2A (PP2A) (Figure 2C) (44). The LT stabilization domain (LSD) is a unique sT domain of MCPyV as being not present in the sT of other HPyVs. The LSD region is involved in inhibiting the proteasomal degradation of LT (45). Unlike other polyomaviruses, MCPyV does not encode both the agnoprotein (46) and the VP3 protein (47).

Lastly, the MCPyV genome encodes for a miRNA precursor that is expressed from the early region. The precursor can produce two mature miRNAs, named MCV-miR-M1-5p and MCV-miR-M1-3p (35,48). Interestingly, MCV-miR-M1 has been found to downregulate the expression of MCPyV LT and restrict viral replication. Moreover, it has also been reported to be required for establishing a long-term persistent infection in MCPyV-infected neuroectodermal tumour PFSK-1 cells (48).

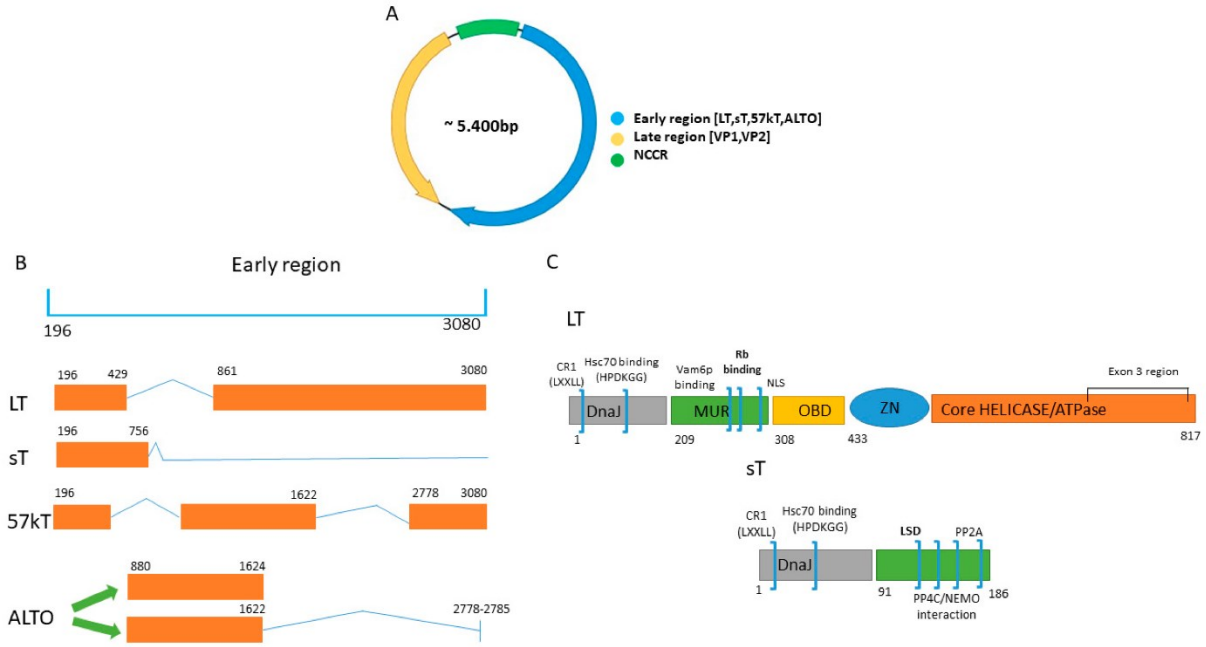


Figure 2. Schematic representation of Merkel cell polyomavirus (MCPyV) genome. Structure of MCPyV genome **(A)**, early region transcripts **(B)** and the early proteins LT and sT with their functional domains **(C).** Adapted from Pietropaolo et al., 2020.

1.3 Detection of Merkel Cell Polyomavirus in humans

Since MCPyV being considered highly tumorigenic, several investigations have been conducted during the past years to evaluate the impact of MCPyV infection in humans. Previous studies have reported the presence of MCPyV DNA in blood and serum from healthy subjects (HS) (49–51). About 14% of peripheral blood mononuclear cells (PBMCs) from pregnant females have been found to be MCPyV DNA-positive (49). Moreover, nearly 2% of sera from HS carry circulating MCPyV DNA (50). MCPyV DNA sequences have also been detected in cutaneous/nasal swabs, eyebrows, chorionic villi and adrenal glands (37,49,52–56). Since being frequently detected in the skin from HS, MCPyV is considered a member of the human skin microbiota (33). Although the lack evidences, MCPyV transmission has been hypothesized to occur via skin-to-skin contact leading to the virions being cutaneously spread (53). Despite MCPyV having high oncogenic potential characteristics, its infection seems to be widespread asymptomatically in different human

populations. Variating results have been reported on MCPyV seroprevalence in HS. Previous immunological studies have revealed widely differing prevalence rates, ranging from 55-87%, according to the study being considered (9,39,65–67,57–64). However, immunological data seems to concordantly indicate that primary MCPyV infection occur early in life (57,61). Nearly all MCPyV-positive MCC (MCCP) patients carry circulating abs against MCPyV (61,68,69).

Current immunological assays for detecting serum IgGs against MCPyV comprise relatively laborious enzyme-linked immunosorbent assays (ELISAs). These assays mainly employ recombinant virus-like particles (VLPs) from MCPyV VP1, as antigens (70). More recent studies have reported the development of multiplex detection assays based on fluorescent bead technology, in combination with a glutathione S-transferase (GST) capture ELISA (71–73). Despite allowing abs against different viruses, including MCPyV, to be detected simultaneously, these systems employ viral capsomers as antigens for immunoreactions (71–73). Using VLPs could be considered a methodological limitation. Indeed, VLPs may increase the probability of cross-reactions between different viruses with a certain degree of homology, therefore hampering the result (67,68,74). In addition, VLP production is a difficult and laborious task, which requires MCPyV VP1 coding sequence cloning and protein *in vitro* synthesis using Spodoptera frugiperda insect cells, or recombinant bacteria. Furthermore, generated VLPs need to be purified and quantified. Only after following these time consuming steps can VLPs be employed as antigens for immunoreactions (57,67,68). On this ground, current VLPs-based immunological assays appear to be expensive, laborious and time consuming (57,67). Given the current state of knowledge, it is important to have specific, rapid, and low-cost methods, which allow abs against MCPyV to be identified unequivocally, in: (i) patients suffering from various diseases, including cancer, such as Merkel Cell Carcinoma (MCC); (ii) immunocompromised organ transplant recipients and AIDS patients; (iii) HS, who can be blood, stem cell and organ donors.

1.4 Merkel Cell Carcinoma

MCC is a rare and highly malignant skin tumour of neuroendocrine origin. MCC has firstly been described by Cyril Toker (75) as a “trabecular carcinoma of the skin with carcinoid features” (75). Subsequently, the presence of neurosecretory granules-membrane-bound granules containing dense cores within the tumour cells has been reported. This feature does not differentiate tumour cells originating from the neural crest and Merkel cells

(76). Based on these findings, the tumour name has been modified in Merkel cell carcinoma to indicate the similarity between tumour and Merkel cells (77–79). Merkel cells can be found in the basal layer of the epidermis, especially around the hair follicles. These cells serve as mechanoreceptors for soft touch stimuli, are associated with afferent sensory nerves and exhibit neuroendocrine characteristics. Neuroendocrine markers, such as chromogranin-A, synaptophysin and the epithelial marker cytokeratin 20 are expressed by these cells (80). These markers are also typically expressed from MCC cells.

In general, MCC presents as an erythematous lesion in the dermal layer which expand rapidly. It is an extremely malignant disease with a high rate of metastasis and recurrence. Indeed, more than one third of MCC-affected patients die from the disease. In addition, MCC often metastasises to draining lymph nodes and distant organs, including pancreas, brain, liver, lungs, and bones (81). MCC has a local recurrence rate ranging between 33-36%, as well as a regional lymph node metastasis rate ranging between 41-55% and a distant metastasis rate ranging between 18-35%. The American Joint Committee on Cancer estimates a 5-year overall survival rate of 51% for local disease, 35% for nodal disease and 14% for metastatic disease (81,82). Although MCC is rare, its incidence is currently about 3/1,000,000 individuals and has increased by more than 95% in the United States since 2000, while the number of cases are continuously raising (83).

The at-risk population for MCC comprise elderly individuals, fair-skinned adults with an extensive sunlight and ultraviolet light (UV) exposure, patients with severe immunodeficiency diseases, including HIV/AIDS, or with medical treatment for autoimmune diseases, solid organ transplantation and other cancers (84). The main features of MCC can be summarised by the acronym AEIOU: asymptomatic/lack of tenderness, expanding rapidly, immunosuppression, older than 50 years, and UV-exposed/fair skin (85).

1.5 Mechanisms of Merkel cell carcinoma tumorigenesis and tumour pathophysiology

Although similar presentation and prognosis, two different MCC aetiologies have been identified. The first, which accounts for the highest proportion (34), ~80%, is caused by MCPyV oncogenic activity (24). The majority of MCC cases are caused by MCPyV infection in the northern hemisphere. On the other hand, an opposite trend has been observed in Australia and New Zealand where MCC cases have been reported to be mostly caused by DNA damages caused by sunlight/UV chronic exposition, without harbouring MCPyV DNA and/or proteins (84,86) (Table 1). The UV exposure as MCC-risk factor may also explain, at least in part, the observation that MCC patients often have a history of other UV-associated

skin cancers, such as basal cell carcinoma or squamous cell carcinoma of the skin (87). UV radiation also induce the expression of inflammatory mediators and functional changes in antigen-presenting dendritic cells, leading to a cascade of events that modulate immune sensitivity (88). It is noteworthy that UV exposure might also play a role in MCPyV-driven carcinogenesis by causing local immunosuppression (89).

Despite important advances in understanding MCC carcinogenesis, the cellular origin of MCC remains unclear. Based on histomorphology, gene expression profiling and molecular analysis, MCC has been hypothesised to originate from Merkel cell progenitors, pre-/pro-B cells (90) or dermal fibroblasts (91). Furthermore, based on the expression of specific neuroendocrine markers, Merkel cells were hypothesized to be the cells of origin of MCC. However, Merkel cells are fully differentiated and do not undergo cell division (92) and there are too few Merkel cells in the skin to account for the millions of copies of MCPyV DNA detected on healthy human skin (53). It might therefore be unlikely that Merkel cells are the cell of origin for MCC. However, further investigations should be conducted to corroborate this conclusion.

Early studies suspected a viral MCC aetiopathogenesis since it had been determined that the MCC incidence resulted as tenfold higher in HIV-1/AIDS patients compared to the general population (93). The risk of developing MCC had also been found to be strongly increased in patients under iatrogenic immunosuppression for the treatment of autoimmune diseases such as rheumatoid arthritis and solid organ transplantation (23,94). Considering that MCC disproportionately affects elderly/immunocompromised and healthy individuals, Huichen Feng and Masahiro Shuda began looking for a pathogenic cause behind the MCC onset (24). To identify possible pathogens, the entire MCC transcriptome was sequenced, and all human genes were removed from the analysis. With this approach, a PyV LT-like transcript was identified, which resulted to be homologous to the known PyV tumour antigens. Complete sequencing of the viral genome in MCC tumours led to the identification of a new HPyV, eventually named MCPyV (24). In 8 out of 10 MCC tumours tested, the viral DNA of MCPyV has been shown to be integrated into the host genome. Moreover, the same restriction fragment length polymorphism pattern has been observed in a primary skin tumour and a metastatic lymph node from the same patient. This suggests that MCPyV was likely causative or at least an early event in MCC tumorigenesis. Since its discovery, MCPyV has been known as a ubiquitous virus that asymptomatically infects different human populations (57,62,95). In the last decade, a large number of studies have demonstrated a causative role for MCPyV in MCC (27,96).

Despite one study reported that almost all MCCs have MCPyV footprints (97), it is currently well accepted that about 80% of all MCC cases are MCCP (24). Consistently, a reduced fraction of MCC tumours is not related to the presence of MCPyV, MCPyV-negative (MCCN), and may instead be caused by a different aetiology (98,99). The two MCC aetiologies also show differences in the expression of diagnostic markers, while a more severe clinical outcome has been described for MCCN than MCCP (100).

Table 1. Features of Merkel cell polyomavirus-positive and -negative Merkel cell carcinoma.

MCPyV-positive MCC	MCPyV-negative MCC
Clonal integration of MCPyV DNA into tumour genome	No presence of MCPyV DNA
Expression of MCPyV small T antigen (ST) and truncated large T antigen (LT)	No expression of MCPyV LT and sT RNA or protein
Wild-type *RB1* and *TP53*	Inactivating mutations in *RB1* and *TP53*
No UV mutational signature	High frequency of DNA mutations induced by UV damage
Predominantly diploid with minimal number of copy number variations	High degrees of aneuploidy
Minimal number of somatic nucleotide alterations	Inactivating mutations in genes involved in various signalling pathways, including DNA damage response and repair genes and chromatin-modifying genes

1.6 Merkel cell polyomavirus-positive Merkel cell carcinoma

MCCP tumours generally presents very few somatic mutations (101–103). The expression of the two MCPyV oncoproteins LT and sT antigens, alongside MCPyV DNA integration into the host genome, are responsible for driving MCCP tumorigenesis. A defining feature of MCCP is that the tumour retains the expression of the early transforming viral genes, i.e., LT and sT (24). Consistently, silencing of MCPyV LT/sT genes in MCCP cell lines resulted in cell death (104).

Clonal integration of the viral genome is characteristic of all MCCP tumours (24,105). The MCPyV genome is also highly mutated when clonally integrated into the host genome (106,107). The NCCR and at least part of the early region are conserved in all MCCPs, including an integral sT and a truncated form of LT (tLT) (34,108). In addition, a C-Ter tLT form is expressed in MCCPs due to nonsense or frameshift mutations in the LT-encoding gene that result in premature stop codons. Tumour-derived tLTs retain the DnaJ

region and the LXCXE motif, also known as retinoblastoma-associated protein (RB1) binding. Contrariwise, the DNA-binding, helicase, and cell growth inhibitory domains required for viral DNA replication are lost (109). The tLT is unable to support viral replication but retains its oncogenic potential (33). In some cases, MCCPs express a tLT that preserves the nuclear localization signal (110,111). Furthermore, these mutants are considered to be required for stable integration of MCPyV genome into the host genome (112), although the mechanism of viral gene integration is still unknown.

Induction of DNA damage by the C-Ter domain of LT stimulates the host DNA damage response, resulting in an indirect activation of p53 and inhibition of cell proliferation (34,113).

Moreover, MCPyV LT contains several potential phosphoacceptor sites and phosphorylation of specific residues in the C-Ter. These post-translational modifications, that occur thought the Ataxia telangiectasia mutated (*ATM*) kinase activity, could induce apoptosis and inhibit proliferation (112,114). Therefore, the LT C-Ter domain exhibits antitumor properties and could explain why this region is deleted in MCCP (113).

RNA interference studies have shown that MCCPs express MCPyV sT. Although its exact molecular functions are not well understood, sT presents a potent oncogenic activity and it is also required for the growth and survival of MCCP cell lines (115). For instance, *in vitro* evidences indicate that sT can transform rat-1 fibroblasts (116) and cooperate with the tLT to transform human fibroblasts (117). Although it seems that sT plays a more dominant role during Merkel cell transformation, LT is important for maintaining the oncogenic phenotype. Specifically, LT overexpression can rescue MCCP cell lines from cell death after T antigen knockdown (115). Expression of sT can lead to significant variations in gene expression, including the overexpression of proglycolytic genes, and can induce aerobic glycolysis in fibroblasts (118). Malignant tumour cells usually have glycolysis rates up to 200-fold higher than their normal cells of origin; phenomenon identified as the Warburg effect. Although sT is able to induce Warburg effect in MCCP cells (118), it is not clear how sT triggers this effect.

Intriguingly, the MCPyV life cycle does not provides the viral integration as most likely being considered a random genetic event (24). Indeed, integrated viral DNA can no longer produce viable virions (34). During MCCP onset, the viral DNA integration into the host genome may occur multiple times, thus providing multiple copies of inserted MCPyV DNA, with each viral genome copy having the same or nearly identical mutations. This observation suggests that the viral DNA might be already mutated before viral DNA integration (34). Moreover, alongside the multiple copies of integrated MCPyV DNA, some

MCCP tumours show that the surrounding cellular DNA is co-amplified with the virus (108,119). These observations have led to a model in which the mutant viral DNA is integrated into the tumour genome, creating a circular DNA form that is subsequently amplifiable by rolling circle amplification before the reinsertion back of the amplified insertion into the host genome (34).

In contrast to MCCNs, MCCP cells generally contain very few mutations, copy number variations and/or signs of UV-mediated DNA damage. In particular, MCCPs have an extremely low number of mutations, which is in the range of 0.4 mutations per megabase (102).

Although both *RB1* and *TP53* are almost never mutated in MCCPs, p53 activity is reduced (120). However, MCPyV tLT does not bind directly to p53, implying that MCPyV sT, tLT or structural changes in the tumour cell genome caused by MCPyV insertion contribute to the reduction of wild-type p53 activity (121). Specifically, the reduction of p53 activity has been linked to E3 ubiquitin-protein ligase (MDM2) activation through mycL/EP400 induced by the sT activity (122). In MCCPs, it is reasonable believe that LT and sT functionally disrupt these signalling pathways, thereby circumventing the need for the respective inactivating mutations.

1.7 Merkel cell polyomavirus-negative Merkel cell carcinoma

The MCCN form is caused by extensive UV-induced mutations and presents a high tumour mutational burden (TMB), often exceeding 20 somatic mutations per mega base (101,102,123). The high frequency of DNA mutations associated with UV damage is also typical of other skin cancers associated with sun exposure, such as melanoma, basal cell carcinoma and cutaneous squamous cell carcinoma (CSCC) of the skin (124). The high levels of UV-associated DNA mutations in MCCN suggests that the cell of origin might be a skin cell exposed to the sun. It is highly likely that MCCN tumours are derived from an epithelial cell of the keratinocyte lineage. One argument against a keratinocytic origin of MCCN is the typical appearance of MCC tumours in the dermal layer. However, several reports of the *in situ* appearance of MCC in association with CSCC have also been reported, thus supporting the possibility that MCCN may originate in the epidermal layer (125–127). A whole-genome sequencing data showed a severely damaged genome with many somatic single nucleotide modifications, translocations, and copy number alterations. Among the genes mutated in MCCN are loss-of-function mutations in the tumour suppressor genes RB transcriptional corepressor 1 (*RB1*) and tumour protein p53 (*TP53*) (128). Mutations that

inactivate *RB1*, which is involved in cell cycle regulation, are frequently found in MCCNs, whereas *RB1* is not mutated in most MCCPs. Furthermore, strong genetic evidence suggests that the target of the tLT is *RB1* (129).

Loss-of-function mutations are also frequent in neurogenic locus notch homologue protein 1 (*NOTCH1*), lysine methyl transferase 2C (*KMT2C*), and lysine methyl transferase 2D (*KMT2D*) in MCCN tumours (119,130). The phosphoinositide 3-kinase (*PI3K*) pathway is activated in MCCN by activating mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) or loss of the inhibitors phosphatase and tensin homolog (*PTEN*), tuberous sclerosis 1 (*TSC1*), and tuberous sclerosis complex 2 (*TSC2*) (102). Furthermore, in addition to the lack of tumour suppressors, the increase of the MYC paralog MYCL (MYCL1 or L-MYC) in MCCN is frequently detected (119,131).

1.8 Merkel cell carcinoma therapeutic approaches

Therapeutic options for MCC are limited (132). Local and nodal MCC can be treated with surgery and radiotherapy, but extensive, metastatic, and recurrent cases of MCC usually require systemic therapy (133). Until the advent of immunomodulatory therapies, cytotoxic chemotherapy based on cisplatin and etoposide was used, which has a high response rate but is limited by its short duration (134). MCC patients are also inclined to develop resistance to therapies, and most develop metastasis. Furthermore, chemotherapy with cisplatin/etoposide is effective but the progression-free survival is improved by ~3 months, only (134). A breakthrough in the treatment of MCC was made when it was discovered that immune checkpoint inhibitor therapy, including anti-programmed death ligand 1 (PD-L1) antibody or anti-programmed death 1 (PD-1) antibody can induce frequent and long-lasting responses, with good results (135–137). However, although PD-1/PD-L1 blockade therapy is promising for cancer treatment (138), a fraction of patients is either non-responder or develop resistance (139). New effective therapeutic options for MCC management are therefore urgently required.

The biological activity of retinoic acid or all-trans retinoic acid (ATRA) is mediated by RAR/RXR receptors, ligand-dependent transcription factors that activate genes involved in differentiation. Without ligands, RAR/RXR suppresses its gene expression (140). Deregulation of RAR/RXR and in particular the receptor β (RARβ) leads to oncogenesis. The inhibitory effect of RARβ on cell growth and potent antineoplastic activity of ATRA have also been demonstrated in several solid tumours (140). Data from several groups

suggest that the RARβ pathway in carcinomas is suppressed epigenetically (141,142). The antitumor pharmacological effect of ATRA in MCC is unknown.

Studies on the clinical application of epigenetic alterations in MCC are limited and mainly focus on histone acetylation. Histone acetylation dysregulations are involved in the MCC host immune-surveillance escape. Similarly to other tumours (143–146), MCC exert a strong immune selective pressure, so it arises and progresses when developing efficient immune escape mechanisms. MCC tumours can evade the antitumour immune response through the epigenetic silencing of human leukocyte antigen (*HLA*) genes and the antigen presentation machinery. There is evidence that histone acetylation plays a role in this process (143,147). Ritter and colleagues reported loss of MHC class I chain-related protein (MIC) A and B expression in MCCP cells (147). This loss has been subsequently described as a consequence of H3K9 deacetylation in near to MIC promoter (147). MIC expression can be epigenetically restored by pharmacological treatment with the histone deacetylases inhibitor (iHDAC) vorinostat (147). Additional cell surface receptors i.e., HLA class-I complex, have been reported as down-regulated in MCC tissues with unknown MCPyV-positivity and in MCCP cell lines (148). The epigenetic re-expression of HLA class-I on the surfaces of MCC cells has been demonstrated by domatinostat , an orally available iHDAC compound (149).

The expression of inhibitory receptors on the anti-tumour immune cells and the surface of tumour cells can be considered another mechanism by which MCC escapes the immune system. During cell transformation, the interaction between PD-1 and PD-L1 expressed on the surface of activated T cells and tumour cells, respectively, reduces T-cell function and prevents the immune system from acting against tumour cells (150). Although the use of PD-1/PD-L1 inhibitors is of remarkable clinical importance for MCC therapy (151), a certain proportion of patients develop pharmacological resistance to the therapy. To circumvent this negative response, iHDAC panobinostat has been administered to a small group of PD-1/PD-L1 non-responder patients with metastatic MCC (152). It has been reported that pharmacological treatment of two patients with panobinostat resulted in HLA class I expression restoration of and in an increase in $CD8^+$ T-cell infiltration in MCC tissues. These data suggest that iHDACs could potentially be helpful in overcoming the loss of HLA class I expression in PD-1/PD-L1 non-responder patients (152).

Dysregulation of histone acetylation in key immune system players is an important mechanism by which MCC can escape the antitumour response, while expression restoration of immunoregulatory complexes by epigenetic priming with iHDACs could be a helpful therapeutic approach based on enhancing the adaptive immune response (153).

Impairment of histone acetylation in MCC involves dysregulation of the proto-oncogene *c-Myc* (154). In MCC tumours/cells, *c-Myc* overexpression has been related to acetylated lysine 27 enrichment at histone H3 (H3K27Ac) in an enhancer proximal to the *c-Myc* promoter. At the same time, high occupancy of Bromodomain protein 4 (BRD4), a gene expression regulator which binds to H3K27Ac has also been reported. In addition, *in vitro* treatment with bromodomain and extraterminal (BET) inhibitors (iBETs), a class of drugs that reversibly bind to BET family proteins, comprising BRD4, depleted BRD4 occupancy at the *c-Myc* enhancer and induced c-Myc down-regulation (154). This evidence underlines not only that both H3K27Ac and BRDs play a role in MCC, but also that iHDAC and iBET combination therapy should be considered as a potentially novel anti-tumour approach. Furthermore, MCPyV sT regulates gene expression via cooperation with MYCL and the EP400 histone acetyltransferase complex (155).

On the other hand, the clinical application of DNA methylation in MCC is less well understood. Epigenetic modifications, such as DNA methylation, are essential for gene regulation. DNA methylation is prone to dysregulation in several types of cancer (140). Improper DNA methylation has been reported to be involved in MCC aetiopathogenesis (132,156). The methylation of promoter of tumour suppressor genes *MGMT, CDKN2A, RASSF1A* and *DUSP2* and the immunoregulatory gene *PD-1* has been reported in several studies (157–161) It has been shown that in MCC both the presence of MCPyV virus and the accumulation of mutations are associated with an accelerated DNA methylation age compared to the chronological age (162).

The biological importance of DNA methylation is underscored also by the clinical efficacy of hypomethylating agents (HMAs) such as azacytidine, decitabine and guadecitabine (163,164), currently in use for the treatment of various haematological malignancies and solid tumours (100,163). MCC exhibits deregulations in DNA methylation (132,156). Also, a recent study conducted on MCC cells reported encouraging data with decitabine (156).

2. Objectives and Aims

2. Objectives and aims of the study

The main objective of the present thesis was to investigate the impact of oncogenic MCPyV infection in the healthy population. To this purpose, a novel immunologic assay was set-up and validated to unequivocally identify circulating IgGs against MCPyV. The need for specific and rapid immunologic assays for MCPyV-seropositivity assessment prompted us to develop and validate a novel indirect ELISA to identify anti-MCPyV IgGs, without using recombinant proteins as antigens.

In a second phase, in order to identify novel therapeutic options for improving MCC management, the antineoplastic drugs ATRA and decitabine were preliminarily evaluated *in vitro* in MCC cells.

2.1 Aim I

Development of a novel indirect ELISA based on two linear synthetic peptides, which mimic MCPyV VP1 and VP2, to unequivocally recognize circulating IgGs elicited against linear/conformational MCPyV VP1 and VP2 mimotopes/epitopes. The indirect ELISA was developed evaluating its performance in detecting serum anti-MCPyV IgGs, considering a number of criteria, such as sensitivity, specificity, efficiency, validity, repeatability/reproducibility and linearity, among other criteria, in a set of controls, i.e., MCPyV-positive and -negative sera. Receiver operating characteristic (ROC) curve analyses were also performed. The assay was then extended, and validated, employing sera from different populations of healthy individuals ranging 0-100 yrs old, including 0-20 yrs old healthy children (HC), 21-65 yrs old healthy subjects (HS) and 66-100 yrs old healthy elderly (ES) subjects. Exposure to MCPyV infection as age/gender-specific MCPyV-seroprevalence and serological profile were therefore determined with the new indirect ELISA.

2.2 Aim II

Preliminary investigation of the antineoplastic effect of ATRA and decitabine on cell proliferation in MCCP cell lines MKL-1 and Peta and in MCCN cell lines MCC26 and MCC13. Since the current MCC therapeutic options being limited, the set-up of new therapies for MCC management is of primary importance to limit the impact of this tumour.

3. Materials and Methods

3. Materials and Methods

3.1 Sera

Human sera **(**n=132) have been tested by indirect ELISAs to evaluate the diagnostic performance of our assay. Indeed, the evaluation was performed on human control sera, which were previously analysed for MCPyV-seropositivity using a VLPs-based ELISA test, as reported (67,68). Controls comprised (i) MCPyV-negative (n=67, mean age ± standard deviation of mean [SD], 53 ± [7] years) and (ii) MCPyV-positive healthy individuals (n=65, mean age ± SD, 50 ± [9] years). Our assay was then validated on: i) n=344 (mean age ± [SD], 9 ± [7] years) sera from healthy children subjects (HC); ii) n=548 (mean age ± [SD], 42 ± [13] years) sera from healthy subjects (HS); iii) n=226 (mean age ± [SD], 78 ± [7] years) sera from healthy elderly subjects (ES) with unknown MCPyV serology taken from our serum collection (165–167). Analyses were conducted within age-stratified HC, i.e., 0-5 (n=138), 6-10 (n=44), 11-15 (n=76), 16-20 (n=86); HS, i.e., 18-30 (n=130), 31-40 (n=120), 41-50 (n=141), 51-65 (n=157); ES, i.e., 66-70 (n=40), 71-75 (n=46), 76-80 (n=57), 81-85 (n=47) and 86-100 (n=36) yrs-old groups.

Furthermore, data from the entire set of sera (n=1,080, mean age ± SD, 40 ± [27] yrs) aged 0-100 yrs, were stratified according to age in 0-5 (n=138), 6-10 (n=44), 11-15 (n=76), 16-20 (n=86), 21-25 (n=39), 26-30 (n=52), 31-35 (n=59), 36-40 (n=61), 41-45 (n=76), 46-50 (n=65), 51-55 (n=50), 56-60 (n=55), 61-65, 66-70 (n=40), 71-80 (n=103), 81-85 (n=47) and 86-100 (n=36) yrs-old groups, and compared.

Serum samples were collected at the Clinical Laboratory Analysis, University Hospital of Ferrara, Ferrara, Italy, from discarded laboratory analysis samples, after routine analyses, before their destruction by incineration. The hospital records indicated these samples are belonging to healthy subjects. Indeed, blood analysis parameters were all in the normal index range. In addition, sera were collected anonymously and coded with indications of age and gender. Written informed consent was obtained from all subjects. The County Ethical Committee, Ferrara, Italy, approved the study (ID:151078). Samples were stored at -80 °C until testing.

3.2 Computational analyses

In silico analyses were performed to assess the reliability of the two linear synthetic MCPyV VP1 S and VP2 F peptides/mimotopes for detecting anti-MCPyV IgGs. Amino acid (a.a.) sequences of VP1 S (24 a.a.) and VP2 F (25 a.a.) peptides, are as follows (Figure 3):

VP1 S: NH_2-NSPDLPTTSNWYTYTYDLQPKGSS-COOH;

VP2 F: NH_2-SLSPTSRLQIQSNLVNLILNSRWVF-COOH.

Sequence analyses/alignments were carried out using the NCBI database and Clustal Omega tool (Hinxton, Cambridgeshire, UK). S and F peptides a.a. sequences were mapped on native MCPyV VPs to verify structural similarities. The monomeric form of MCPyV VP1 was obtained from the Protein data Bank (PDB, ID:4FMG) (168), while MCPyV VP2 was obtained via computational prediction carried out using the DNASTAR tool (Lasergene, Madison, WI). Molecular VPs visualizations were performed using the DNASTAR tool.

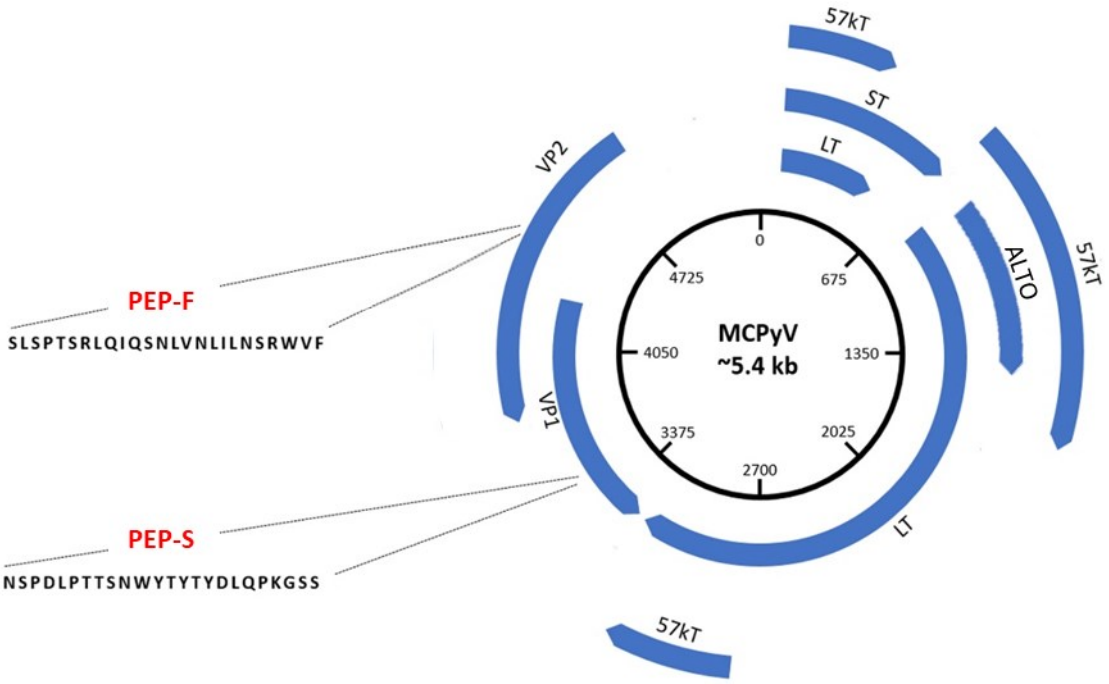


Figure 3. Schematic representation of the MCPyV genome. MCPyV DNA consists of two regions: the early region and the late region. The early region contains sequences coding for early genes, including the Large T antigen (LT), the small T antigen (sT) and the 57 KT protein. The late region contains sequences coding for late genes, including the proteins VP 1 and VP 2. The peptides PEP-S and PEP-F, which are used as specific antigens in indirect ELISA tests to detect IgG and IgM antibodies against MCPyV, are highlighted in red.

3.3 Indirect ELISA

The indirect ELISA was developed and validated to detect specific IgGs against MCPyV. The initial concept of the assay has been reported for other polyomaviruses (PyVs) (29), while the herein newly employed assay was specifically conceived for MCPyV.

3.3.1 Peptide coating

S and F peptides were purchased from UFPeptides s.r.l., Ferrara, Italy. ELISA plates (Nunc-immuno plate, Thermo Fisher, Milan, Italy) were coated with 5 μg of selected peptide for each well, diluted in 100 μL of Coating Buffer 1X (Candor Bioscience, Wangen, Germany). Plates were left at 4°C for 16 h and then rinsed three times with Washing Buffer (Candor Bioscience, Wangen, Germany).

3.3.2 Peptide blocking

Blocking phase was performed using 200 μL/well of blocking solution (Candor Bioscience, Wangen, Germany) at 37°C for 90 min (169–171). Wells were rinsed three times.

3.3.3 Primary antibody adding

Each well was covered with 100 μL containing 1:20 diluted sera in low cross-buffer (Candor Bioscience, Wangen, Germany). Control sera in each plate were: (i) positive controls, three immune sera derived from patients with MCPyV-positive MCC; (ii) negative controls, three MCPyV-negative sera. Subsequently, plates were rinsed three times, before adding secondary Ab min (169–171).

3.3.4 Secondary antibody adding

A goat anti-human IgG heavy (H) and light (L) chain specific peroxidase conjugate (Calbiochem-Merck, Darmstadt, Germany) or peroxidase-labelled affinity purified antibody to human IgM heavy chain μ peroxidase-conjugate (KPL Gaithersburg MD, USA) were diluted 1:10,000 in Low Cross-Buffer. The reaction mixture was incubated at room temperature (RT) for 90 min (169–172).

3.3.5 Dye treatment and spectrophotometric reading

Wells were rinsed three times, and then 100 μL of 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution (Sigma-Aldrich, Milan, Italy) was added to each well. After 45 min at RT plates were read by spectrophotometer (Thermo Electron Corp., model Multiskan EX, Vantaa, Finland) at a wavelength (λ) of 405 nm. Colour intensity in wells was determined by optical density (OD) reading.

3.3.6 Cut-off determination

The cut-off of each peptide was determined in each ELISA run, as the mean of the OD readings of n=3 negative control sera, adding three standard deviations of mean (mean +3 SDs) (173,174), as described for other ELISAs (165,169–171,175,176). Sera were considered MCPyV-positive when reacting to both S and F synthetic peptides, in three replica experiments carried out by independent operators.

3.4 Indirect ELISA performance evaluation

Indirect ELISA performance in detecting serum anti-MCPyV IgGs was evaluated on MCPyV-negative (n=67) and MCPyV-positive (n=65) control sera. The following sample conditions were considered: true positive (TP, the number of positive samples according to previous analyses), false positive (FP, the number of positive samples with our assay and negative with previous analyses), true negative (TN, the number of negative samples according to previous analyses) and false negative (FN, the number of negative samples with our assay and positive with previous analyses) (177). Assay performance was then assessed computing a number of set-up criteria (177–182), as follows: sensitivity (Se, TP/[TP+FN]), specificity (Sp, TN/[TN+FP]), positive predictive value (PPV, TP/[TP+FP]), negative predictive value (NPV, TN/[TN+FN]), validity (as [Se+Sp]/2), accuracy (Se*Prevalence+Sp*[1−Prevalence] and the overall efficiency (Ef) (or relative agreement, as [TP+TN]/[TP+TN+FP+FN] (Table 2) (177). Assay accuracy was evaluated considering the following combined measures: Youden's Index (J, Se+Sp-1) (178), positive likelihood ratio (LR+, as Se/[1-Sp]), negative likelihood ratio (LR-, [1-Se]/Sp) (176,177). Furthermore, Cohen's kappa coefficient (κ) was used to evaluate the agreement between expected and obtained results (180). The agreement was interpreted as poor ($\kappa \leq 0$), slight ($0 < \kappa \leq 0.20$), fair ($0.21 < \kappa \leq 0.40$), moderate ($0.41 < \kappa \leq 0.60$), substantial ($0.61 < \kappa \leq 0.80$), and near-perfect ($0.81 < \kappa \leq 1.0$) (180). The performance of each S and F peptide was also analysed by building receiver-operating characteristic (ROC) curves (183). ROC curves were used to calculate the area under the curve (AUC) for each peptide by comparing expected/obtained results (181). The AUC provides a global summary of statistic test robustness by considering the assay as: non-informative (AUC=0.5), low ($0.5 < AUC \leq 0.7$), moderate ($0.7 < AUC \leq 0.9$) and high ($0.9 < AUC < 1$) accurate and perfect (AUC=1) (182). Concordance in ODs between S and F peptides was evaluated on MCPyV-negative/-positive control sera using Spearman correlation r coefficient (with the 95% CI) (184).

3.5 Determining indirect ELISA precision and dilutional linearity

Indirect ELISA precision was assessed by evaluating assay repeatability (intra-assay variability) and reproducibility (inter-assay variability). Intra-assay repeatability was evaluated by measuring the coefficient of variations (CVs) for 90 repeats of 30 sera for each MCPyV VP1 S and VP2 F peptide. Sera with high (n=10, OD>0.17 for S peptide and OD>0.4 for peptide F), medium (n=10, 0.12<OD<0.17 for S peptide and 0.2<OD<0.4 for F peptide) and low (n=10, OD<0.12 for S peptide and OD<0.2 for F peptide) ODs were selected. Inter-assay reproducibility was evaluated by performing 3 independent runs for each peptide, by different operators. Adequate rates were determined for intra-assay CV to 10% and inter-assay CV to 15% (185). In order to determine assay dilutional linearity (accuracy), 3 samples with high ODs (obtained with the assay used herein) and diluted 1:20, 1:40 1:80, 1:160, 1:320, 1:640, 1:1,280 and 1:2,560 were selected and tested in triplicate.

3.6 Merkel cell carcinoma cell lines

The MCC cell lines MCPyV-positive, i.e., PeTa and MKL-1 (European Collection of Authenticated Cell Cultures [ECACC] ID: 09111801), and -negative, i.e., MCC-13 (ECACC ID: 10092302) and MCC-26 (ECACC ID: 10092304) (186), were cultured with RPMI 1640 medium containing 10% foetal bovine serum (FBS, EuroClone) (156).

3.7 Pharmacological treatments

All-trans retinoic acid (ATRA, Merck) and 5-aza-2'-deoxyazacitidine (Decitabine, DAC, Merck) were dissolved in DMSO and H_2O, respectively, at a concentration of 100 mM as stock solution. Untreated and 0.1% DMSO treated MCC cell lines were used as controls. Pharmacological treatments begun 24 hours after the cell seeding. ATRA was administered over a 3-day period, while the HMA drug decitabine was administered over a 5-day period (141). Moreover, ATRA was administered on the first treatment day, only, while decitabine was refreshed daily (Figure 4). Experiments were performed in triplicate.

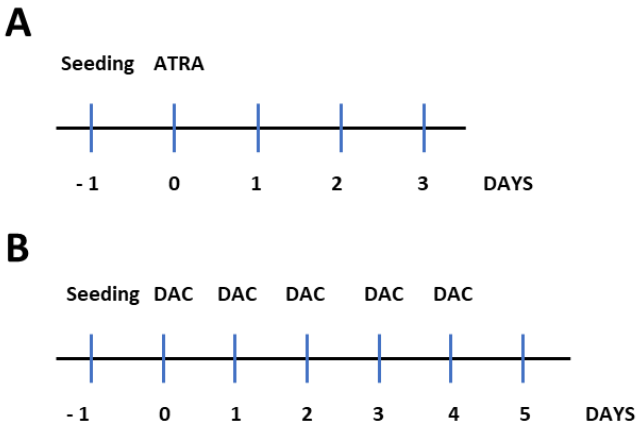


Figure 4. Treatment schedule. MCC cells were treated according to the scheme, incubation with one ATRA pulse **(A)** or five decitabine 24h pulses **(B).** In the latter case, medium and drugs were removed and replaced with fresh medium to which the drugs were added. Cell proliferation was evaluated in MCC cell lines using WST-1 assay (1-3 and 1-5 day- schedule for ATRA and decitabine, respectively).

3.8 Cell proliferation

Following pharmacological treatments, the cell proliferation was assessed using the WST-1 assay (Roche) in 96-well plates (140,156). Untreated MCC cells and DMSO-treated MCC cells, were used as controls. Half-maximal inhibitory concentration (IC_{50}), i.e., the value representing the minimal compound concentration required for a 50% inhibition of the cell proliferation, was calculated by measuring cell proliferation by WST-1 assay (187).

3.9 Statistical analysis

A two-sided chi-square test was used to statistically analyse MCPyV seroprevalences (167,188). Values were analysed using the D'Agostino Pearson normality test and then parametric and non-parametric tests were applied according to normal and non-normal variables, respectively (184,189). Linear regression of correlation coefficient (R^2) (with the 95% CI) was computed to evaluate the ELISA assay linearity (184). Data were analysed using the one-way Anova analysis and Kruskal-Wallis multiple comparison test (OD mean, 95% CI). Statistical analyses were carried out using MedCalc Statistical Software version 16.2.1 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org) and Graph Pad Prism version 8.0 for Windows (Graph Pad, La Jolla, USA) (190,191). P values <.05 were considered statistically significant (192,193).

4. Results

4. Results

4.1 MCPyV peptides selected by computer assisted analyses

Two linear synthetic peptides, named MCPyV VP1 S and VP2 F (S and F peptides), which mimic the immunoreactive MCPyV VP1 and VP2 epitopes, respectively, were designed and employed in indirect ELISA as mimotopes to detect anti-MCPyV IgGs. The identity between a.a. strains from S and F peptides and corresponding VP native polyomaviruses (PyVs) from known 16 human/simian PyVs, including MCPyV and its different strains, was assessed (Figure 5A-D). The S and F peptides a.a. sequences were 100% concordant with MCPyV VP1 and VP2 strains, respectively (Figure 5B, D). The S peptide a.a. sequence was 100% identical to the corresponding VPs from the five main MCPyV isolates, including North American (MCC350 or EU375803.1 and MCC339, or EU375804.1), Japanese (TKS and FJ464337), European (MKL-1 or FJ173815), and Chinese (HB039C or KC571692.1) isolates (23,194). Likewise, the F peptide a.a. chain was 100% identical to that of VPs from North American and European isolates (23,194). Moreover, the a.a. sequence of F peptide was 96% (24/25 a.a.) concordant with that of VPs from the Japanese and Chinese isolates (F to S at the last a.a. position of the peptide, n.25) (23,194), as well as with that from AHW79949 and AWG42110 VP strains (T to I at a.a. position n.5 of the peptide in both cases) (195,196).The identity with the VP native strains from other 15 PyVs was below 37.5% and 36% for S and F peptide, respectively. In addition, a total of 12 and 8 different PyV types shared less than 16.6% and 16% of identity with the a.a. strains from S and F peptides, respectively (Figure 5B, D). Both S and F peptides also differ from VP native strains from 15 PyVs, due to the frequent presence (or absence) of additional a.a. residues within their sequence (Figure 5B, D).

Computational analyses indicate that S peptide corresponds to a.a. 66-89 of MCPyV VP1, while F peptide lies within a.a. 153-177 of MCPyV VP2. In addition, S peptide also encompasses the VP1 BC surface loop, an important immunoreactive site. Indeed, the natural immune response against MCPyV VP1 is mainly directed against the BC surface loop (197). Notably, a.a. sequences from S and F peptides were characterized toward a stable secondary structure formation. Indeed, S peptide forms a stable secondary structure from a.a. 8 to a.a. 11, i.e., $_{8}$TSNW$_{11}$, where an alpha helix domain is found, surrounded by two random coil secondary structures (Figure 5E). F peptide has 3 random coil secondary structures, while it presents three short beta sheet domains from a.a. 1 to a.a. 2, i.e., $_{1}$SL$_{2}$, from a.a. 18 to a.a. 19, i.e., $_{18}$IL$_{19}$, and from a.a. 22 to a.a. 23, i.e., $_{22}$RW$_{23}$ (Figure 5E). Tertiary structures of MCPyV VPs were retrieved from the available PDB structures (VP1) and computationally

predicted by using the DNASTAR tool (VP2). The three-dimensional graphic rendering of S and F peptides mapped onto the inferred MCPyV VP1 and VP2 native structures, respectively, indicated that these regions are exposed on VP surfaces (Fig. 5F, G) (197). The spatial configuration of S and F peptides may thus represent natural short docking sites for serum IgGs against both linear and conformational antigens.

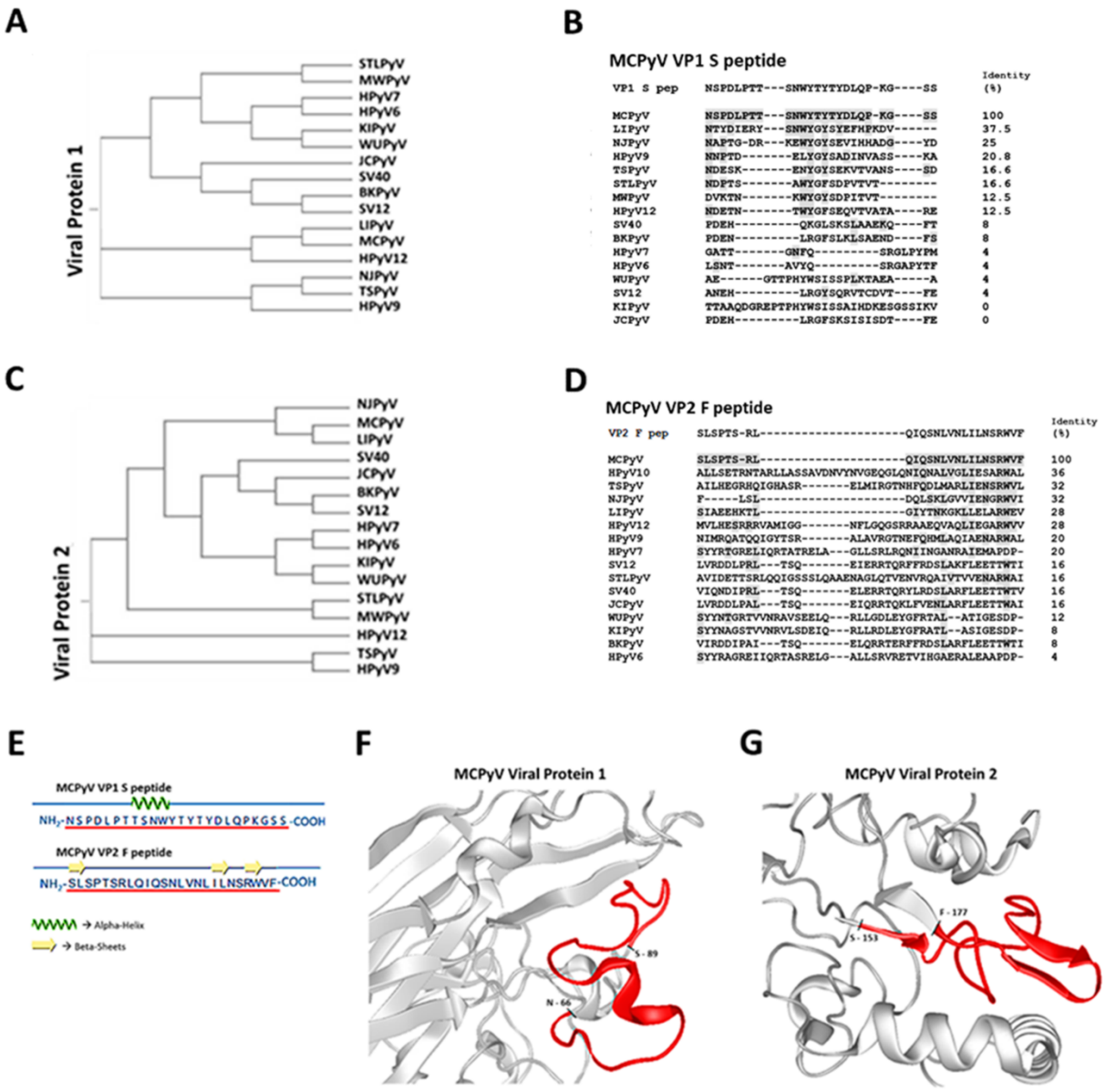


Figure 5. Amino acid sequences of S/F peptides, polyomaviruses phylogenetic trees and partial VP1/2/3 structures visualizations. Panels (**A**) and (**C**) show PyVs phylogenetic trees according to VP1 and VP2 from human/simian PyVs comparisons. Panels **(B)** and (**D)** show identity between Merkel cell polyomavirus (MCPyV)-specific VP1 S and VP2 F peptides and VP1 and VP2 from human/simian polyomaviruses (PyVs). Both S and F peptides were selected based on their low homology with corresponding a.a. sequences from VP1 and VP2 from known human/simian PyVs. VP1 and VP2 a.a. sequences/IDs were obtained from https://www.ncbi.nlm.nih.gov/protein/ and aligned with Clustal Omega software. Panel **(E)**: computational analyses indicate that both peptides show several random coiled domains with 1 alpha helix (S peptide) and 3 beta-sheets (F peptide). Panels **(F)** and **(G):** partial view of S and F peptides (red) within the three-dimensional (3D) models of VP1 and VP2. The partial VP2 representation is based on a structural prediction obtained using DNASTAR software (169).

4.2 Indirect ELISA performance parameters evaluation

Developing our indirect ELISA provided for an evaluation of its performance in detecting serum anti-MCPyV IgGs on MCPyV-negative (n=67) and -positive (n=65) 1:20 diluted control sera. The evaluated criteria, computed by comparing expected and obtained results, are depicted in Table 2. The assay demonstrated 80.00% (95% CI: 68.23–88.89%) sensitivity and 91.05% (95% CI: 81.52–96.64%) specificity, with a PPV of 94.32% (95% CI: 88.45-97.29) alongside a NPV of 71.03% (95% CI: 59.97-80.03%), in detecting serum anti-MCPyV IgGs (Table 2). Likewise, the efficiency, validity and accuracy criteria resulted as 85.61%, 85.52% and 83.87% (95% CI: 76.46-89.68%), respectively (Table 2). The evaluation also indicated that J, LR+ and LR- were 0.71, 8.93 (95% CI: 4.12-19.35) and 0.22 (95% CI: 0.13-0.35), respectively (Table 2). Agreement between expected and obtained results resulted as 85.60%, with a κ of 0.72 (95% CI: 0.61-0.84) (Table 2).

Table 2. Indirect ELISA diagnostic performance criteria.

Criteria	Values
Sensitivity (Se)	80.00%
Specificity (Sp)	91.05%
Positive Predictive Value (PPV)	94.32%
Negative Predictive Value (NPV)	71.03%
Efficiency (Ef)	85.61%
Validity	85.52%
Accuracy	83.87%
Youden's Index (J)	0.71
Likelihood ratio (LR+)	8.93
Likelihood ratio (LR-)	0.22
Agreement	85.60%
Cohen's Kappa value (κ)	0.72

Indirect ELISA diagnostic performance criteria obtained by testing MCPyV-negative (n=67) and -positive (n=65) control sera (169).

ROC curves were built based on ODs obtained on MCPyV-positive and -negative control sera, for both S and F peptides. AUC resulted as 0.821 (95% CI: 0.745 to 0.882) for the peptide S and 0.738 (95% CI: 0.654 to 0.811) for the peptide F (Figure 6). The difference between AUCs for both S and F peptides was statistically significant compared to that of a worthless test (AUC=0.5, P<0.001) (Figure 6). ODs obtained with S and F peptides on same control sera were also compared. A good correlation between ODs for S and F peptides was found to be, using a Spearman correlation coefficient r, 0.8723 (p<0.0001) (Figure 6C).

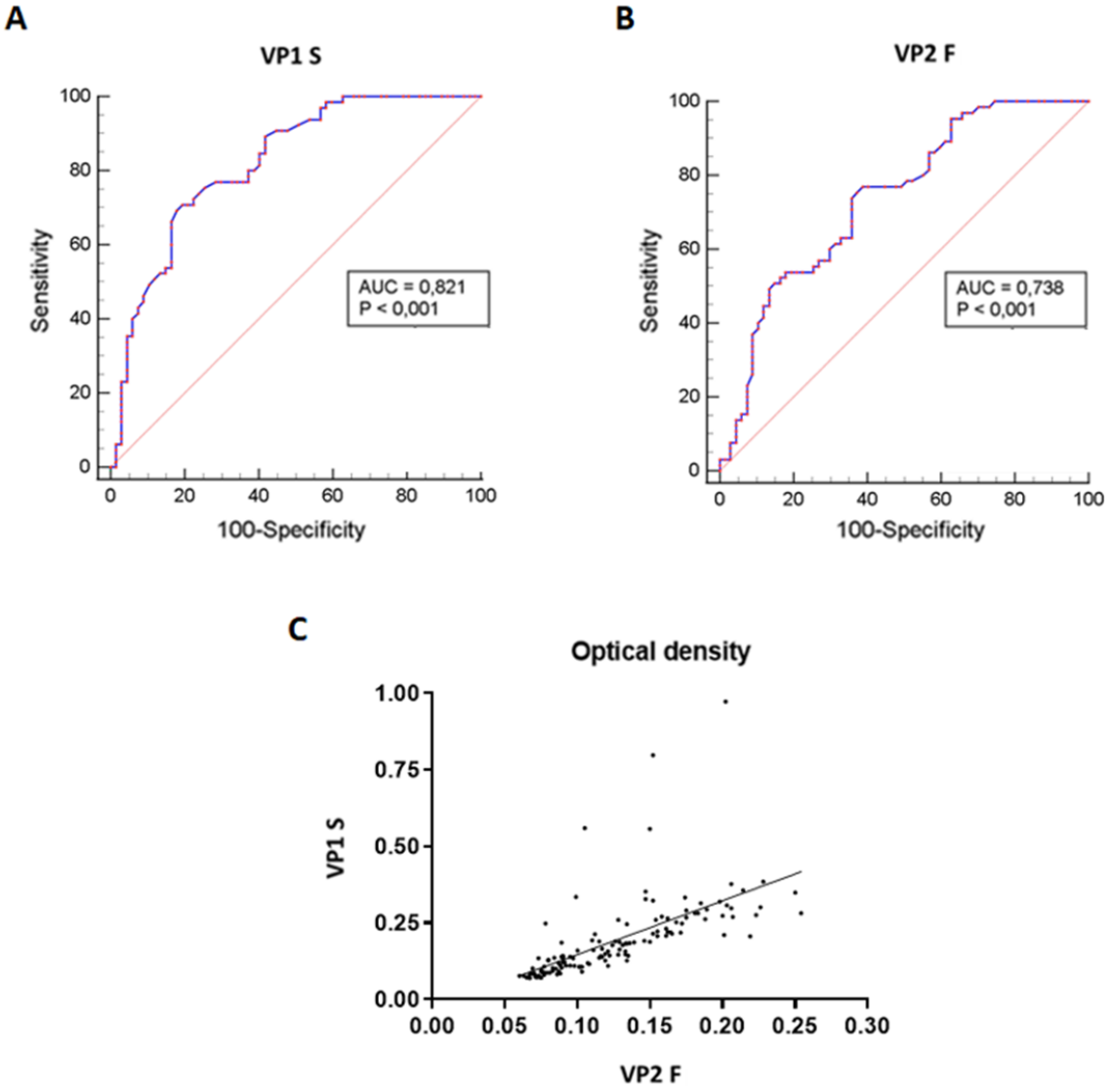


Figure 6. Receiver-operator characteristic (ROC) curves and correlation of Optical density (OD) values of the indirect ELISA. ROC curves were built based on optical density (OD) values obtained on MCPyV-negative (n=67) and -positive (n=65) control sera, for both MCPyV VP1 S and VP2 F peptides. The values for the area under the ROC curve (AUC) were 0.821 for VP1 S peptide **(A)** and 0.738 for VP2 F peptide **(B)**. The diagonal line shows an AUC value of 0.5 which is representative of a worthless test. The difference between AUCs for both S and F peptides resulted statistically significantly different from that of a worthless test (AUC=0.5, P<0.001). **(C)** The concordance in ODs between the S and F peptides was evaluated on the entire set of MCPyV-negative/-positive control sera (n=132) using Spearman correlation analysis. Concordance between VP1 S peptide and VP2 F peptide was good, with an r of 0.8723 and a p<0.0001 (169).

4.3 Indirect ELISA repeatability, reproducibility and dilutional linearity evaluation

To assess indirect ELISA precision in terms of repeatability (intra-assay variability) and reproducibility (inter-assay variability), CVs were measured on n=30 MCPyV-positive sera which were tested in triplicate in one run and in three independent experiments, for each S and F peptide (Table 3). Sera were selected and stratified according to the ODs obtained with both peptides in sera with low (n=10), medium (n=10) and high (n=10) ODs. A total of 180 repeats were done.

Intra-assay repeatability CVs for low, medium, and high OD groups were 5.03%, 7.28% and 7.45% for S peptide, respectively, and 6.41%, 6.02% and 7.17% for F peptide, respectively (Table 3). Inter-assay reproducibility CVs were 4.40% and 5.65% for the low OD group, 7.09% and 8.05% for the medium OD group and 7.86% and 8.85% for the high OD group, for S and F peptide, respectively (Table 3).

Table 3. Indirect ELISA intra-assay and inter-assay coefficient of variations (CVs).

Peptides	**Groups**		
	Low (n=10)	**Medium** (n=10)	**High** (n=10)
S peptide			
Intra-assay (n=3 replicates)			
Mean	0.062	0.156	0.178
SD	0.006	0.051	0.057
CV (%)	5.03	7.28	7.45
Inter-assay (n=3 replicates)			
Mean	0.063	0.161	0.186
SD	0.005	0.049	0.06
CV (%)	4.40	7.09	7.86
F peptide			
Intra-assay (n=3 replicates)			
Mean	0.077	0.259	0.57
SD	0.021	0.087	0.21
CV (%)	6.41	6.02	7.17
Inter-assay (n=3 replicates)			
Mean	0.078	0.267	0.592
SD	0.022	0.092	0.216
CV (%)	5.65	8.05	8.85

Indirect ELISA intra-assay and inter-assay coefficient of variations (CVs) evaluated on three set of control sera with high (n=10), medium (n=10) and low (n=10) optical density levels, for both Merkel cell polyomavirus VP1 S and VP2 F peptides. SD, standard deviation; CV, coefficient of variation (169).

Dilutional linearity (accuracy) of the indirect ELISA was determined by performing serial dilutions, from 1:20 to 1:2,560, (i) using n=3 MCPyV-seropositive samples with known high ODs when tested using both S and F peptides; (ii) on three sets of MCPyV-seropositive samples (n=15) previously studied (169), with known high (n=5), medium (n=5), and low (n=5) ODs.

In both cases, dilutions were assayed in triplicate, and ODs/dilution values were compared by linear regression analysis.

In the first evaluation, the assay had a high correlation between ODs and sample dilutions when the S peptide was employed, with an R^2 of 0.9781 ($p<0.0001$), 0.9925 ($p<0.0001$) and 0.9809 ($p<0.0001$) for samples #1, #2 and #3, respectively (Figure 7A). The assay also showed a high correlation between ODs and sample dilutions when using F peptide, with an R^2 of 0.9793 ($p<0.0001$) for sample #1, 0.9851 ($p<0.0001$) for samples #2 and 0.9853 ($p<0.0001$) for samples #3 (Figure 7B).

Results from the second evaluation indicate that the assay had a high correlation between ODs and sample dilutions when the S peptide was employed, with an R^2 of 0.9786 ($p<0.0001$), 0.9959 ($p<0.0001$) and 0.9768 ($p<0.0001$) for samples with high, medium, and low ODs, respectively (Figure 7C). The assay also showed a high correlation between ODs and sample dilutions when F peptide was used, with an R^2 of 0.9773 ($p<0.0001$), 0.9784 ($p<0.0001$), 0.9846 ($p<0.0001$) for samples with known high, medium, and low ODs (Figure 7D).

Moreover, in order to assess the reliability of the immunoassay, ODs obtained with S and F peptides on HC and ES sera were compared by Spearman correlation analysis. OD concordance between S and F peptides for the whole set of HC sera (n=344) was evaluated. Results indicate a very strong correlation between ODs for S and F peptides, with a Spearman coefficient r of 0.8381 ($p<0.0001$) (Figure 7E). Moreover, a good concordance in OD between MCPyV S and F peptides was also determined for the entire set of ES sera (n=226). Results reveal a good degree of correlation between ODs for S and F peptides, with a Spearman coefficient r of 0.7991 ($p<0.0001$) (Figure 7F).

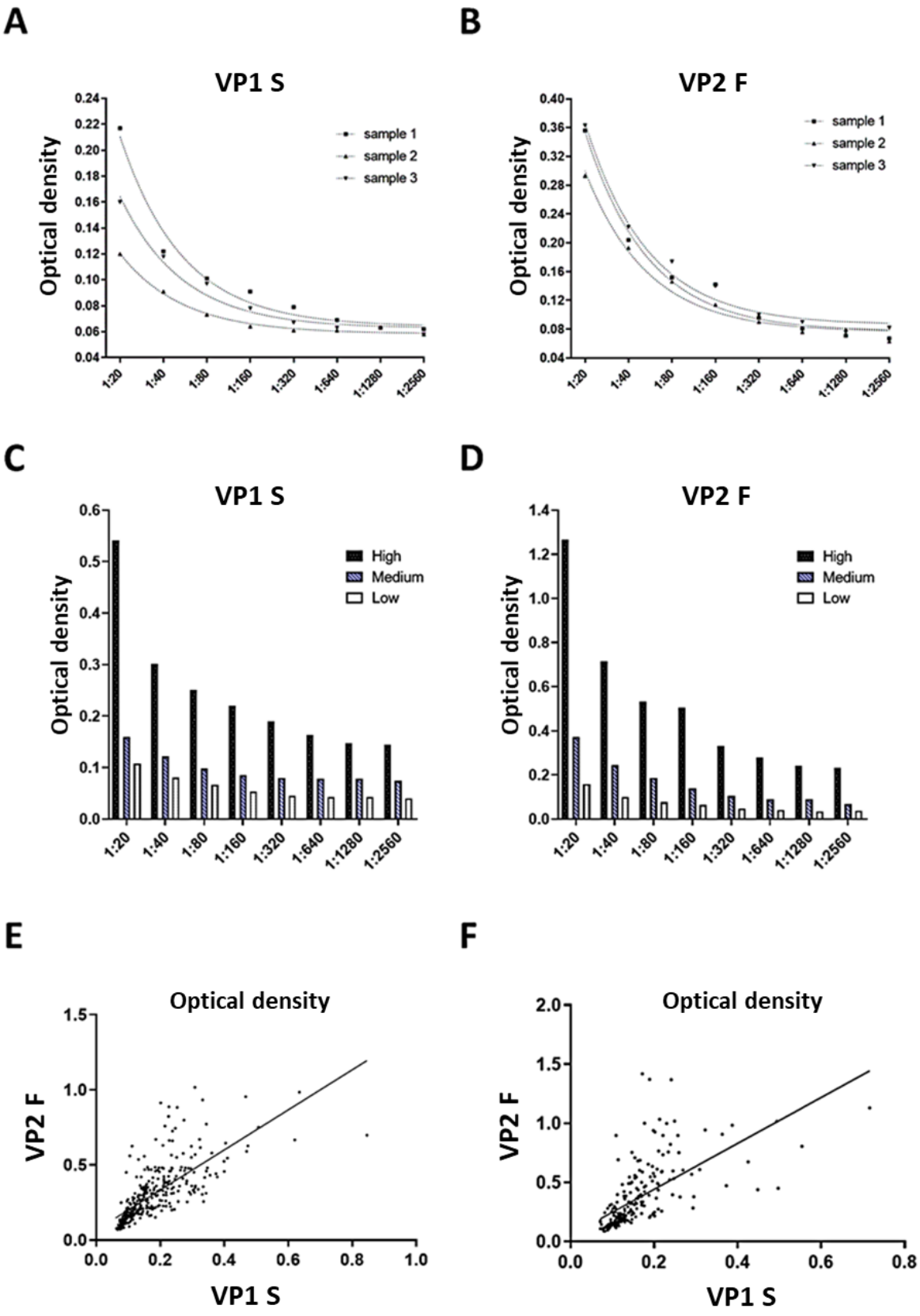


Figure 7. Dilutional linearity correlation of Optical density (OD) values of the indirect ELISA. Optical Density (OD) readings at λ 405 nm, in response to serial dilutions (1:20, 1:80, 1:160, 1:320, 1:640, 1:1,280 and 1:2,560) of n=3 MCPyV-positive sera presenting known high ODs. Each dilution was assayed in triplicate for each MCPyV VP1 S and VP2 F peptide, and ODs and sera dilutions were compared by linear regression analysis. (**A)** Good correlation between ODs and dilutions was

found for VP1 S peptide with an R^2 of 0.9781 ($p<0.0001$), 0.9925 ($p<0.0001$) and 0.9809 ($p<0.0001$) for samples #1, #2 and #3, respectively. (**B)** Good correlation between ODs and dilutions was found for VP2 F peptide, with an R^2 of 0.9793 ($p<0.0001$) for sample #1, 0.9851 ($p<0.0001$) for samples #2 and 0.9853 ($p<0.0001$) for samples #3. **(C)** Optical Density (OD) response to serial dilutions (1:20, 1:80, 1:160, 1:320, 1:640, 1:1,280 and 1:2,560) was evaluated on n=15 MCPyV-seropositive sera with known high (n=5), medium (n=5), and low (n=5) ODs. Each dilution was assayed in triplicate for each MCPyV VP1 S and VP2 F peptide, and OD values and sera dilutions were compared by linear regression analysis. High correlation between ODs and dilutions was found for VP1 S peptide with an R^2 of 0.9786 ($p<0.0001$), 0.9959 ($p<0.0001$) and 0.9768 ($p<0.0001$) for samples with high, medium, and low ODs, respectively. **(D)** Good correlation between ODs and dilutions was found when VP2 F peptide was employed, with an R^2 of 0.9773 ($p<0.0001$) for sample with known high ODs, 0.9784 ($p<0.0001$) for samples with medium ODs and 0.9846 ($p<0.0001$) for samples with low ODs. **(E)** ODs concordance between the S and F peptides was evaluated on the entire set of healthy children (HC) (n=344) using Spearman correlation analysis. A very strong correlation between ODs for S and F was determined, with an r with an r of 0.8381 ($p<0.0001$). **(F)** The concordance in ODs between the VP1 S and VP2 F peptides was evaluated also on the entire set of sera from elderly subjects (ES) (n=226) using Spearman correlation analysis. A good correlation between S peptide and F peptide was found, with an r of 0.7991 and a $p<0.0001$ (169).

4.4 Serum antibodies reacting to Merkel cell polyomavirus VP1 and VP2 mimotopes in healthy children

4.4.1 Detection of serum IgG antibodies against Merkel cell polyomavirus by indirect ELISA in healthy children

The developed indirect ELISA, based on VP1 S and VP2 F peptides/mimotopes, was designed to determine whether serum samples from HC (n=344) contain IgG Abs reactive to MCPyV antigens and to determine the distribution of MCPyV serology in HC. Sera from HC reacting to S and F peptides achieved an overall similar prevalence of 47.7% (164/344) and 51.5% (177/344), respectively (Table 4, $p>0.05$).

Table 4. Seroprevalence of IgG antibodies reacting with Merkel cell polyomavirus VP1 S and VP2 F peptides in heathy children (HC).

			Number of positive samples (%)		
Age yrs	**Number of samples**	**Male (%)**	**VP1 S**	**VP2 F**	**VP1 S+VP2 F**
0-5	138	56.5	29 (21)	30 (21.7)	18 (13)*
6-10	44	43.2	25 (56.8)	27 (61.4)	23 (52.3)
11-15	76	44.7	52 (68.4)	55 (72.4)	46 (60.5)
16-20	86	33.7	58 (67.4)	65 (75.6)	53 (61.6)
Total	344	46.5	164 (47.7)	177 (51.5)	140 (40.7)

*Serum samples (n=344) were from healthy children aged 0-20 years old (HC). Statistical analyses were performed using the two-sided chi-square test. *The different prevalence of MCPyV antibodies evaluated in 0-5 yrs age-stratified group was statistically significant compared with the cohorts aged 6-10 yrs, 11-15 yrs and 16-20 yrs ($p<0.0001$).*

Furthermore, negative sera for the S peptide did not react with the F peptide. Only a few serum samples were exceptions. Indeed, 10.8% (37/344) of sera were negative for S peptide while reacting positively to F peptide. Similarly, 7% (24/344) of sera were negative for F while positive for S peptide ($p>0.05$). When MCPyV-positive sera were combined, for both VP1 S and F peptides, the overall prevalence in HC was 40.7% (140/344). Combined S and F peptides reactivity was then determined in age-stratified HC, i.e., 0-5 yrs, 6-10 yrs, 11-15 yrs and 16-20 yrs, and rates were compared. In the HC cohorts aged 0-5, 6-10, 11-15 and 16-20 years, a prevalence pattern of combined S and F resulted as 13% (18/138), 52.3% (23/44), 60.5% (46/76) and 61.6% (53/86), respectively (Table 4). Notably, a significantly lower seroprevalence was found in the cohort aged 0-5 yrs compared to all the remaining age-stratified cohorts (Figure 8, $p<0.0001$).

In order to investigate a correlation between MCPyV infection and gender in HC, the presence of Abs against MCPyV was determined in HC males (n=160) and females (n=184) and then rates were compared. It was found that the prevalence of anti-MCPyV Abs was similar in males and females, 37.5% (60/160) and 42.3% (80/184), respectively ($p>0.05$).

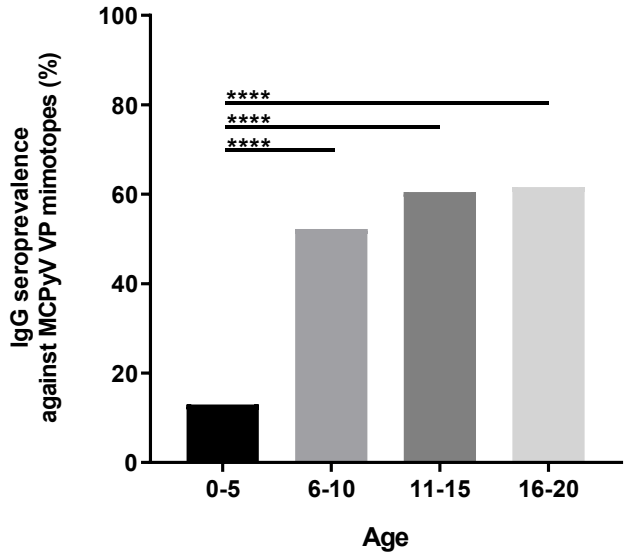


Figure 8. Seroprevalence of anti-Merkel cell polyomavirus IgG antibodies among age-stratified healthy children (HC). Seroprevalences were compared in age-stratified HC i.e., 0-5 yrs, 6-10 yrs, 11-15 yrs and 16-20 yrs. Statistical analyses were performed with two-sided chi-square test. The prevalence detected in 0-5 yrs group resulted significantly lower compared to 6-10 yrs, 11-15 yrs and 16-20 yrs groups ($p<0.0001$).

4.4.2 Serological profiles of IgG reacting to Merkel cell polyomavirus in healthy children

Serological profiles of IgG Abs reacting to VP1 S and VP2 F peptides were analysed, both for specific peptide and in combination. Immunological data were taken from the entire HC cohort (n=344) and results are reported as OD readings at λ 405 nm. The median (interquartile range [IQR]) ODs for S and F peptides, both for specific mimotope and in

combination, were then determined in age-stratified HC and values were compared. The median (IQR) OD values for S peptide resulted as 0.1 (0.08-0.15), 0.21 (0.16- 0.26), 0.18 (0.14-0.24), and 0.21 (0.14-0.26) in age-stratified HC groups aged 0-5, 6-10, 11-15, 16-20 yrs old, respectively (Figure 9A). The median (IQR) OD values for F peptide resulted as 0.16 (0.11-0.25), 0.32 (0.22- 0.46), 0.33 (0.24-0.42), and 0.33 (0.22-0.46) in age-stratified HC groups aged 0-5, 6-10, 11-15 and 16-20 yrs old, respectively (Figure 9B). The median (IQR) ODs for combined S and F peptides were 0.13 (0.09-0.19), 0.23 (0.19- 0.36), 0.24 (0.16-0.35) and 0.24 (0.17-0.35) in age-stratified HC groups aged 0-5, 6-10, 11-15, 16-20 yrs old, respectively (Figure 9C). The median OD values for S and F peptides individually and in combination were significantly lower in the cohort aged 0-5 yrs compared to the other age-stratified cohorts ($p<0.0001$ Figure 9A-C).

To assess any gender differences in the HC group, the median (IQR) ODs for S peptide and F peptide, both for specific peptide and in combination, were also determined in HC males (n=160) and females (n=184), while values were compared. The median (IQR) ODs for S and F peptides in the male group were 0.15 (0.1-0.2) and 0.24 (0.16-0.36), respectively, while in the female group they were 0.16 (0.11-0.24) and 0.26 (0.18-0.42), respectively (Figure 9D, E $p>0.05$). Furthermore, the median (IQR) ODs for combined S and F peptides were evaluated in males and in females. Higher median (IQR) ODs for combined S and F peptides were found in females 0.21 (0.13-0.33) than in males 0.18 (0.12-0.3) ($p<0.05$) (Figure 9F).

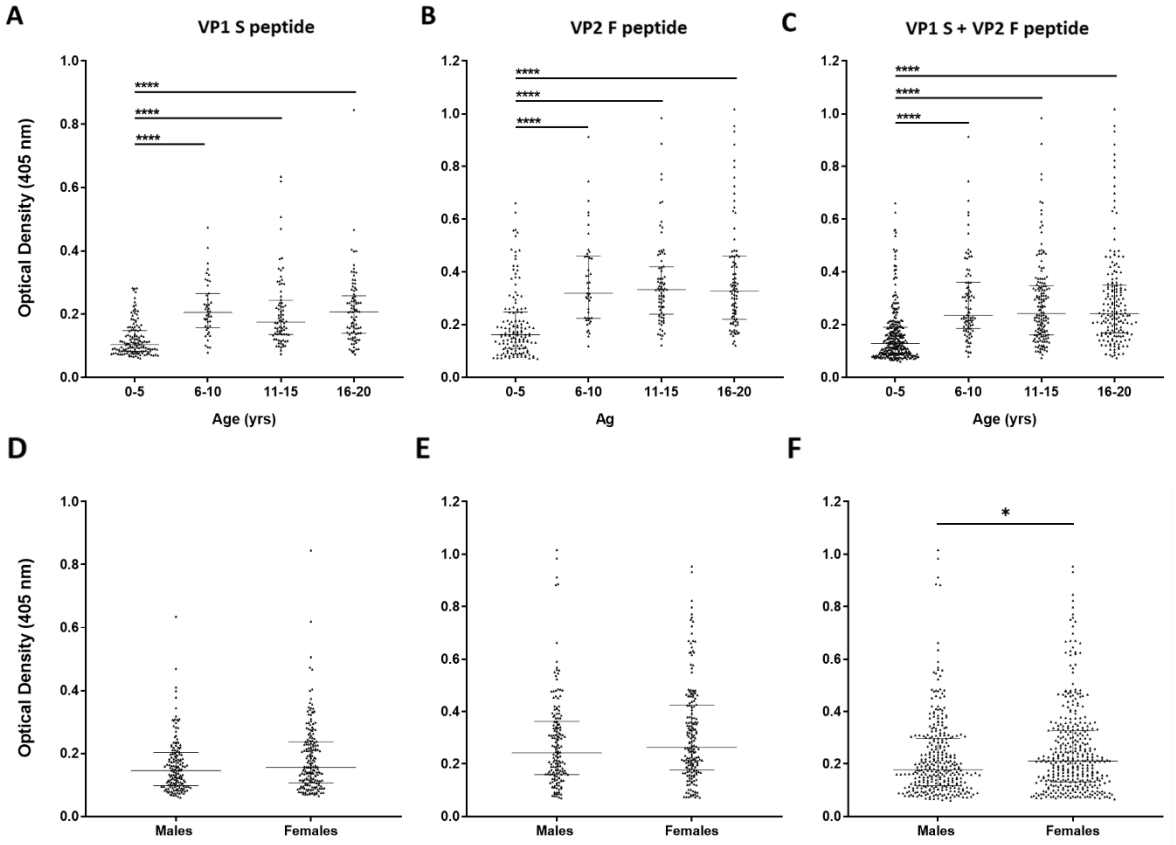


Figure 9. Serological profiles of serum IgG antibody reactivity to Merkel cell polyomavirus VP1 S (A, D), VP2 F (B, E) peptides and for combined S and F peptides (C, F) in age- and gender-stratified healthy children (HC). Immunologic data are from age- and gender-stratified HC and results are reported as optical density (OD) value readings at λ 405 nm for serum samples assayed in indirect ELISA. In the scatter dot plot, each dot represents the dispersion of ODs for each sample. The median is indicated by the line inside the scatter plot with the interquartile range (IQR) in age-stratified HC, i.e., 0-5 (n=138), 6-10 (n=44), 11-15 (n=76) and 16-20 (n=86) yrs and in gender-stratified HC, i.e., males (n=160) and females (n=184). Panels **(A), (B)** and **(C)** ****p<0.0001 for 0-5 *vs* 6-10, 0-5 *vs* 11-15, and 0-5 *vs* 16-20; **(F)** *p<0.01.

4.4.3 Detection of serum IgM antibodies against Merkel cell polyomavirus by indirect ELISA in healthy children

The indirect ELISA employing S and F peptides/mimotopes was employed to evaluate whether serum samples from HC (n=344) contain IgM Abs react to MCPyV antigens and to determine the distribution of the early stage of MCPyV exposition in HC. Sera from HC reacting to S and F peptides reached an overall equal prevalence of 39.5% (136/344) and 39.5% (136/344), respectively (Table 5, p>0.05). Moreover, sera resulted negative for the S peptide were also negative for the F peptide, with only a few serum samples as exception. In fact, 10% (34/344) of sera were negative for S peptide but reacted positively to F peptide. Similarly, 10% (34/344) of sera were negative for F, while positive for S peptide (p>0.05). The combination of MCPyV-positive sera, for both S and F peptides,

resulted in an overall prevalence of 29.7% (102/344) in HC. The combined S and F peptide reactivity was then determined in age-stratified HC, i.e., 0-5 years, 6-10 years, 11-15 years and 16-20 years, while rates were compared. The prevalence pattern of combined S and F peptides corresponded to 29% (40/138), 29.5% (13/44), 32.9% (25/76), and 27.9% (24/86), in the cohorts of HC aged 0-5, 6-10, 11-15 and 16-20 yrs old, respectively (Table 5, $p>0.05$).

To determine an association between MCPyV infection and gender, the presence of Abs against MCPyV was determined in HC males (n=160) and females (n=184) and then the rates were compared. Interestingly, the prevalence of anti-MCPyV-Abs was found to be higher in females, 34.2% (63/184), than in males, 24.4% (39/160) ($p<0.05$).

Table 5. Seroprevalence of IgM antibodies reacting with Merkel cell polyomavirus VP1 S and VP2 F peptides in heathy children (HC).

			Number of positive samples (%)		
Age yrs	**Number of samples**	**Male (%)**	**VP1 S**	**VP2 F**	**VP1 S+VP2 F**
0-5	138	56.5	46 (33.3)	54 (39.1)	40 (29)
6-10	44	43.2	18 (40.9)	16 (36.4)	13 (29.6)
11-15	76	44.7	37 (48.7)	29 (38.2)	25 (32.9)
16-20	86	33.7	35 (40.7)	37 (43)	24 (27.9)
Total	344	46.5	136 (39.5)	136 (39.5)	102 (29.7)

Serum samples (n=344) were from healthy children (HC) aged 0-20 yrs old. Statistical analyses were performed using the two-sided chi-square test. No statistical differences were detected among age-stratified groups ($p>0.05$).

4.4.4 Serological profiles of IgM reacting to Merkel cell polyomavirus in healthy children

The serological profiles of IgM Abs reactivity to MCPyV immunoantigens were examined for S and F peptides, both for specific peptide and in combination. Immunological data became from the entire HC cohort (n=344) and results are reported as OD readings at λ 405 nm. The median (IQR) ODs for VP1 S and VP2 F peptide, both for individual mimotopes and in combination, were then determined in age-stratified HC and values were compared. The median (IQR) OD values for S peptide resulted as 0.1 (0.07-0.23), 0.32 (0.23-0.43), 0.32 (0.24-0.4), and 0.2 (0.16-0.27) in age-stratified HC groups aged 0-5, 6-10, 11-15, 16-20 yrs old, respectively (Figure 10A). ODs for S peptide were found to be lower in HC aged 0-5 than in the remaining age-stratified groups (0-5 yrs *vs* 6-10 yrs, 0-5 yrs *vs* 11-15 yrs, $p<0.0001$; 0-5 yrs *vs* 16-20 yrs, $p=0.0005$; Figure 10A). In addition, lower OD values

were also detected in HC aged 16-20 yrs compared to 6-10 yrs and 11-15 yrs groups (16-20 yrs vs 6-10 yrs, $p<0.0008$; 16-20 yrs vs 11-15 yrs, $p<0.0001$, Figure 10A).

The median (IQR) OD values for F peptide resulted as 0.11 (0.07-0.27), 0.41 (0.32-0.53), 0.27 (0.21-0.36), and 0.2 (0.16-0.25) in age-stratified HC groups aged 0-5, 6-10, 11-15, 16-20 yrs old, respectively (Figure 10B). Furthermore, lower levels of OD for F peptide were detected in sera from HC aged 0-5 yrs than in the remaining age-stratified groups (0-5 yrs vs 6-10 yrs, 0-5 yrs vs 11-15 yrs, $p<0.0001$; 0-5 yrs vs 16-20 yrs, $p<0.05$, Figure 10B). In addition, ODs were higher in the group aged 6-10 yrs then in the cohorts aged 11-15 yrs and 16-20 yrs (6-10 yrs vs 11-15 yrs, $p<0.05$; 6-10 yrs vs 16-20 yrs, $p<0.0001$). Lower ODs were also detected in HC aged 16-20 yrs compared to 11-15 yrs groups ($p<0.01$, Figure 10B).

The median (IQR) ODs for combined S and F peptides resulted to be 0.1 (0.07-0.26), 0.37 (0.25-0.48), 0.29 (0.22-0.4) and 0.2 (0.16-0.26) in age-stratified HC groups aged 0-5, 6-10, 11-15, 16-20 yrs old, respectively (Figure 10C). Moreover, lower ODs for combined S and F peptides were detected in 0-5 yrs group than in the remaining age-stratified groups (0-5 yrs vs 6-10 yrs, 0-5 yrs vs 11-15 yrs, 0-5 yrs vs 16-20 yrs, $p<0.0001$; Figure 10C). Lower OD values were also detected in HC aged 16-20 yrs compared to 6-10 yrs and 11-15 yrs groups ($p<0.0001$, Figure 10C).

Median (IQR) ODs for S and F peptides, both for individual peptides and in combination, were then determined in HC males (n=160) and females (n=184), and values were compared. The ODs for S and F peptides in males were 0.19 (0.1-0.3) and 0.19 (0.11-0.3), respectively, while resulting lower than those found in females, which were 0.24 (0.14-0.38) and 0.24 (0.15-0.39), respectively ($p<0.01$, Figure 10D, E). In addition, the median (IQR) ODs for combined S and F peptides were 0.18 (0.12-0.3) in males and 0.24 (0.15-0.39) in females (Figure 10F). Similarly, the male group showed lower OD values than the female group for the combination of S and F peptides ($p<0.0001$, Figure 10F).

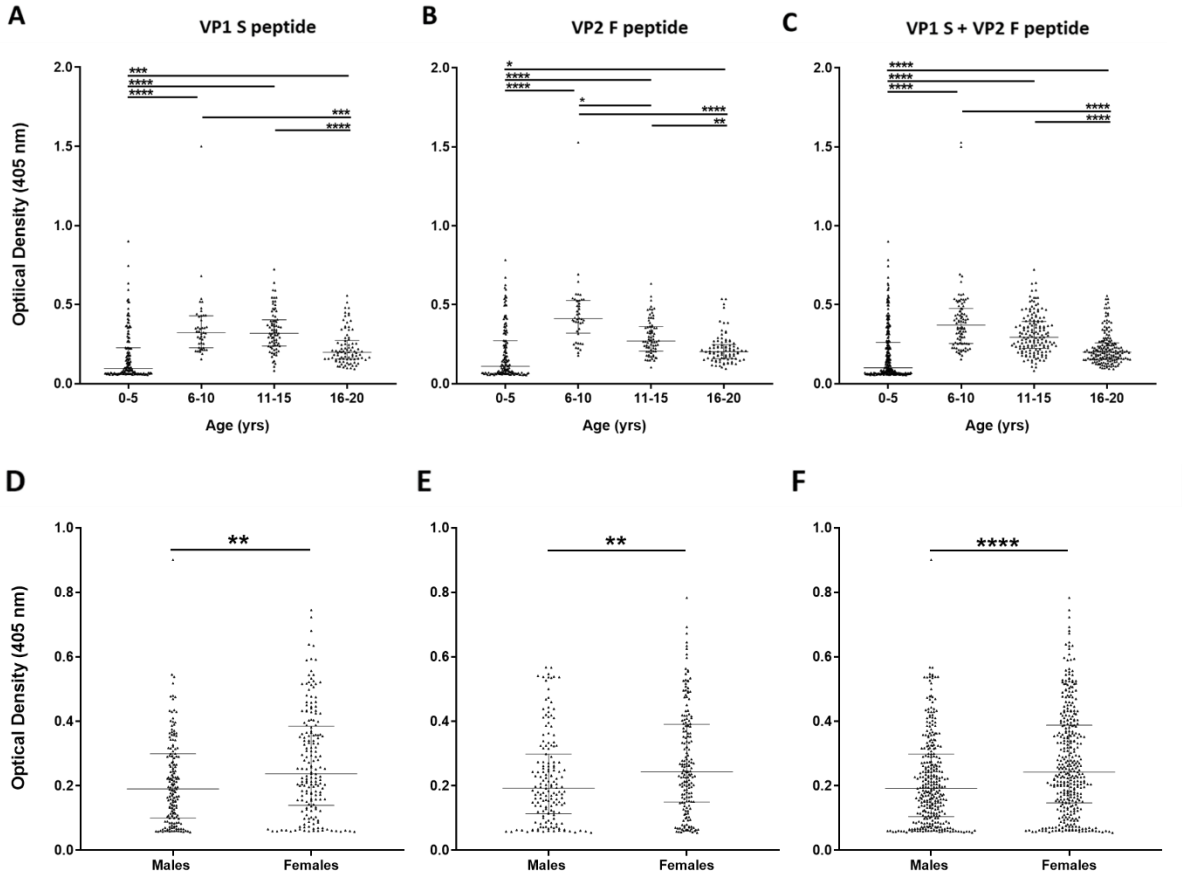


Figure 10. Serological profiles of serum IgM antibody reactivity to Merkel cell polyomavirus VP1 S (A, D), VP2 F (B, E) peptides and for combined S and F peptides (C, F) in age- and gender-stratified healthy children (HC). Immunologic data are from age- and gender-stratified HC and results are reported as optical density (OD) value readings at λ 405 nm for serum samples assayed in indirect ELISA. In the scatter dot plot, each dot represents the dispersion of ODs for each sample. The median is indicated by the line inside the scatter plot with the interquartile range (IQR) in age-stratified HC, i.e., 0-5 (n=138), 6-10 (n=44), 11-15 (n=76) and 16-20 (n=86) yrs and in gender-stratified HC, i.e., males (n=160) and females (n=184). **(A)** ****p<0.0001, ***p<0.001; **(B)** ****p<0.0001, **p<0.01, *p<0.05; **(C)** ****p<0.0001. Panels **(D)** and **(E)** *p<0.001; **(F)** ****p<0.0001.

4.4.5 Relationship between circulating IgM and IgG antibodies to MCPyV in healthy children throughout ages

The entire cohort of HC sera (n=344) was then investigated for determining a possible relationship between serum anti-MCPyV IgG and IgM. Serological data of IgM and IgG Abs reactivity were therefore examined for S and F peptides, both for specific peptide and in combination, in 2-yr age-stratified HC and plotted. Results are reported as median OD readings at λ 405 nm.

The IgM levels for S peptide peaked at 0.36-0.33 OD (median OD range) within 12-14 yrs, whilst being followed by a continuous decline until reaching the median value of 0.18 OD observed in the older HC group aged 20 yrs old (Figure 11A). Regarding the IgG

levels, they remained relatively constant throughout ages until 10-12 yrs where a continuous increase was afterwards found. Indeed, the highest median IgG levels peak of 0.23 OD (median OD range) was determined at 20 yrs (Figure 11A).

A similar pattern was observed in for F peptide, where a median IgM levels peak of 0.41-0.49 OD (median OD range) was reached within 8-10 yrs and followed by a continuous decrease until reaching the median value of 0.17 OD determined at 20 yrs (Figure 11B). Contrariwise, IgG levels remained relatively constant throughout ages eventually reaching the highest level of median 0.35 OD at 20 yrs (Figure 11B).

Consistently, results indicate that the pattern throughout ages did not vary substantially when combining median OD values of S and F peptides, both for IgMs and IgGs. Indeed, a median IgM levels peak of 0.39-0.43 (median OD range), for combined S and F peptides, was observed within 8-10 yrs, whilst being followed by a continuous decrease until reaching the median value of 0.18 OD determined in the older HC group aged 20 yrs old (Figure 11C). In contrast, the median IgG levels remained relatively constant throughout ages, while peaking at 20 yrs where a median value of 0.3 OD was determined Figure 11C).

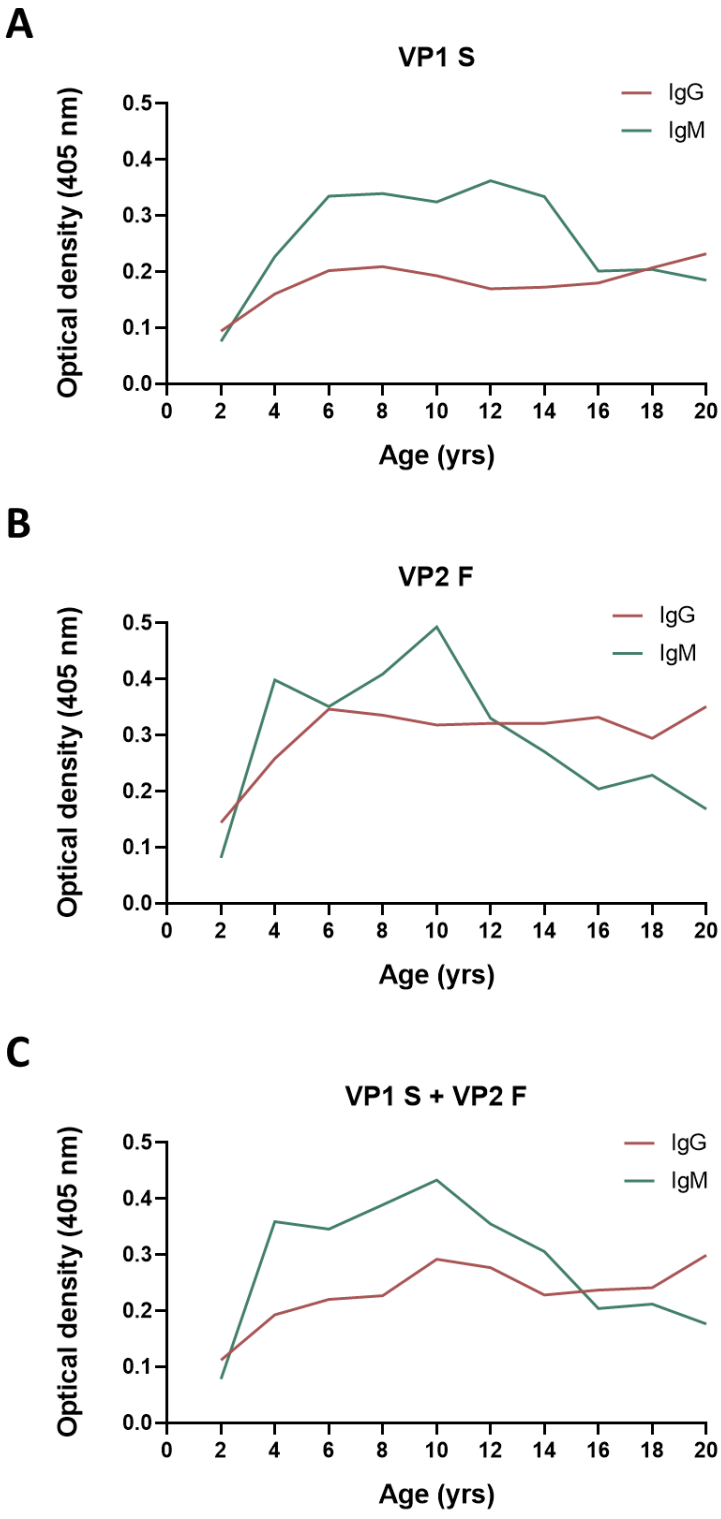


Figure 11. Representative patterns of circulating IgM and IgG antibodies to Merkel cell polyomavirus in 2 yr age-stratified healthy children (HC). Analyses were performed with VP1 S **(A)**, VP2 F **(B)** peptides and for combined VP1 S and VP2 F **(C)**. Immunologic data are from age-stratified HC (n=344) and results are reported as optical density (OD) value readings at λ 405 nm for serum samples assayed in indirect ELISA. MCPyV IgM and IgG patterns were reported as green line and red line, respectively.

4.5 Serum IgG antibodies reacting to Merkel cell polyomavirus VP1 and VP2 mimotopes in healthy subjects

4.5.1 Detection of serum IgG antibodies against Merkel cell polyomavirus by indirect ELISA in healthy subjects

The novel indirect ELISA employing S and F peptides/mimotopes was developed to assess whether serum samples from HS (n=548) contain IgG Abs which react to MCPyV antigens and to determine the distribution of MCPyV infection in HS. Sera from HS which react to S and F peptides reached an overall similar prevalence, of 72.4% (397/548) and 67.8% (372/548), respectively (Table 6, $p>0.05$). Conversely, negative sera for the S peptide failed to react with F peptide. Few serum samples were exceptions. Indeed, 4.9% (27/548) of sera were negative for S peptide, while testing positive for F peptide. Similarly, 9.5% (52/548) of sera resulted negative for F, while testing positive for S peptide. Combining MCPyV-positive sera, both for S and F peptides, overall prevalence in HS was 62.9% (345/548). Combined S and F peptides reactivity was then determined in age-stratified HS, i.e., 18-30 yrs, 31-40 yrs, 41-50 yrs, and 51-65 yrs, and rates were compared. A prevalence pattern of combined S and F corresponding to 63.1% (82/130), 56.7% (68/120), 64.5% (91/141), and 66.2% (104/157), was found in cohorts of HS aged 18-30, 31-40, 41-50 and 51-65 yrs old, respectively (Table 6, $p>0.05$).

To assess any association between MCPyV infection and gender, the presence of Abs against MCPyV were determined in males (n=210) and females (n=338) HS, then rates were compared. It turned out that there was a similar prevalence of anti-MCPyV Abs in both males and females which resulted as 65.2% (137/210) and 61.5% (208/338) ($p>0.05$), respectively.

Table 6. Seroprevalence of IgG antibodies reacting with Merkel cell polyomavirus VP1 S and VP2 F peptides in heathy subjects (HS).

			Number of positive samples (%)		
Age yrs	**Number of samples**	**Male (%)**	**VP1 S**	**VP2 F**	**VP1 S+VP2 F**
18-30	130	20.8	92 (70.8)	90 (69.2)	82 (63.1)
31-40	120	36.7	79 (65.8)	72 (60)	68 (56.7)
41-50	141	39	104 (73.8)	102 (72.3)	91 (64.5)
51-65	157	53.5	122 (77.7)	108 (68.8)	104 (66.2)
Total	548	38.3	397 (72.4)	372 (67.8)	345 (62.9)

Serum samples (n=548) were from healthy subjects (HS). Statistical analyses were performed using the two-sided chi-square test. No statistical differences were detected among age-stratified groups (p>0.05) (169).

4.5.2 Serological profiles of IgG reacting to Merkel cell polyomavirus in healthy subjects

Serological profiles of serum IgG Abs reactivity to S and F peptides alone and to combined S and F peptides are shown in Figure 12. Immunological data are from the entire cohort of HS (n=548) whereas results are presented as OD readings at λ 405 nm. The dispersion of ODs is reported in the scatter dot plot, where each dot represents one OD for each serum sample tested (Figure 12). Median (IQR) ODs were then determined in age-stratified HS, i.e., 18-30 yrs, 31-40 yrs, 41-50 yrs, and 51-65 yrs and compared.

The median (IQR) ODs for S peptide resulted as higher in HS aged 41-50 yrs (0.15, 0.12-0.22) than in those aged 31-40 yrs (0.13, 0.11-0.19, $p<0.05$) (Figure 12A). The median (IQR) ODs for F peptide were higher in sera from both HS aged 51-65 yrs (0.27, 0.21-0.44) and 41-50 yrs (0.31, 0.20-0.47) than in those from 31-40 yrs (0.23, 0.17-0.34, $p<0.05$, Figure 12B). Furthermore, higher median (IQR) ODs for combined S and F peptides were detected in 41-50 yrs group (0.21, 0.15-0.34) than in 31-40 yrs group (0.18, 0.13-0.26, $p<0.01$, Figure 12C).

Serum Ab reactivity to S and F peptides was also evaluated in gender-stratified HS (Figure 12D-F). Median (IQR) OD values were therefore determined in males (n=210) and females (n=338) and compared. The median (IQR) ODs for S peptide in males (0.15, 0.12-0.2) resulted higher compared to those in the female group (0.13, 0.1-0.21, $p<0.05$, Figure 12D). Similarly, median (IQR) ODs for F peptide were higher in sera from males (0.28, 0.21-0.42) than in those from females (0.24, 0.17-0.40, $p< 0.01$, Figure 12E). Consistently, higher median (IQR) ODs for combined S and F peptides were detected in males (0.21, 0.15-0.3) than in females (0.19, 0.12-0.29, $p<0.01$, Figure 12F).

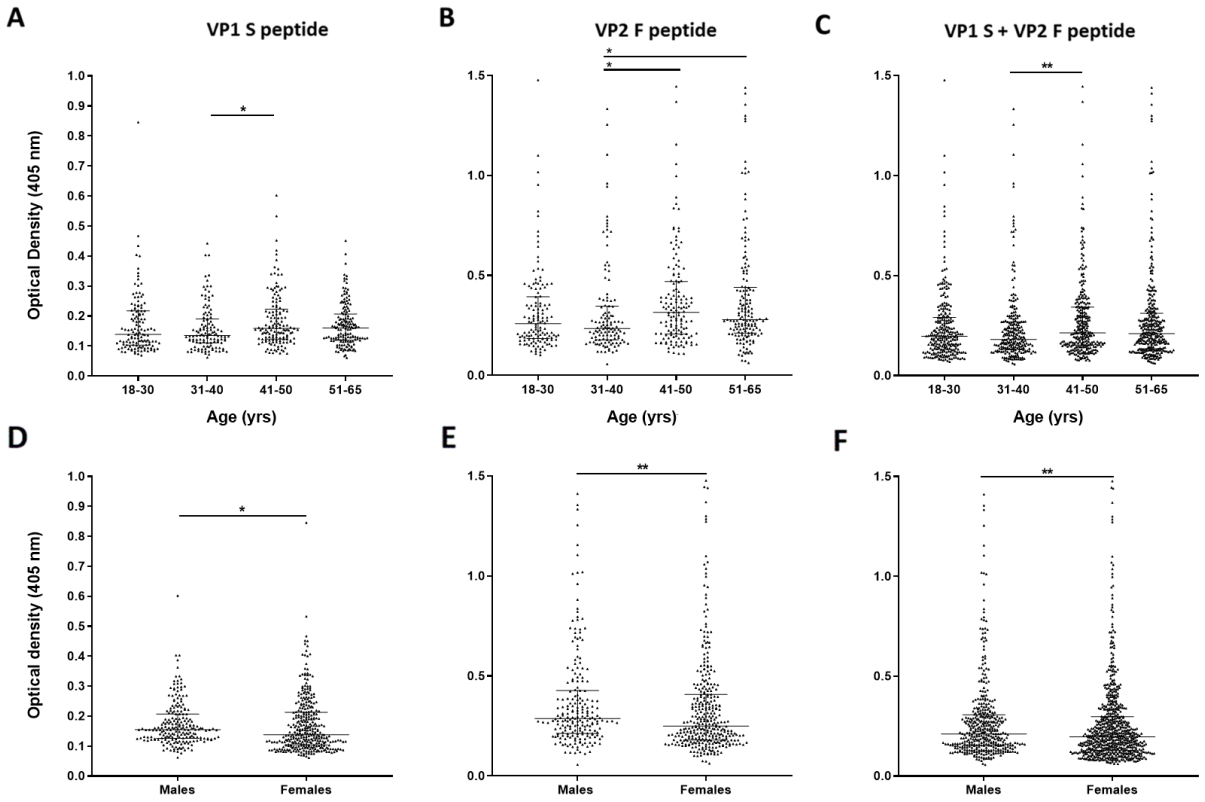


Figure 12. Serological profiles of serum IgG antibody reactivity to Merkel cell polyomavirus VP1 S (A, D), VP2 F (B, E) peptides and for combined S and F peptides (C, F) in age- and gender-stratified healthy subjects (HS). Immunologic data are from age- and gender-stratified HS and results are reported as optical density (OD) value readings at λ 405 nm for serum samples assayed in indirect ELISA. In the scatter dot plot, each dot represents the dispersion of ODs for each sample. The median is indicated by the line inside the scatter plot with the interquartile range (IQR) in age-stratified HS, i.e., 18-30 yrs (n=130), 31-40 yrs (n=120), 41-50 yrs (n=141), and 51-65 yrs (n=157), and in gender-stratified HS, i.e., males (n=210) and females (n=338) **(A)** *p<0.05; **(B)** *p<0.05; **(C)** **p<0.01; **(D)** *p<0.05; **(E)** **p<0.01; **(F)** **p<0.01 (169).

4.6 Serum IgG antibodies reacting to Merkel cell polyomavirus VP1 and VP2 mimotopes in healthy elderly subjects

4.6.1 Detection of serum IgG antibodies against Merkel cell polyomavirus by indirect ELISA in healthy elderly subjects

In order to assess the distribution of MCPyV infection in elders, sera from ES (n=226) were tested by indirect ELISA for IgG Abs reactivity to MCPyV VP1 S and VP2 F peptides/mimotopes. A similar overall prevalence of 67.7% (153/226) and 71.2% (161/226) was obtained in sera from ES reacting to S and F peptides, respectively (p>0.05) (Table 7).

Moreover, with a few exceptions, sera testing negative for S peptide did not react to F peptide. In detail, 7.5% (17/226) of sera resulting negative for S peptide were positive for F peptide, while 3.9% (9/226) of sera were negative for F, while being positive for S peptide. The combined overall prevalence, for both S and F peptides, in ES sera resulted as 63.7%

(144/226) (Table 7). Serum reactivity to MCPyV was then determined in age-stratified ES and rates were compared. A total of 62.5% (25/40), 71.7% (33/46), 64.9% (37/57), 63.8% (30/47) and 52.8% (19/36) of serum samples were found to be positive for IgG Abs reacting to MCPyV in ES cohorts aged 66-70, 71-75, 76-80, 81-85 and 86-100 yrs old, respectively (Table 7). No statistically significant differences in MCPyV seroreactivity were determined among groups ($p>0.05$).

To evaluate an association between MCPyV infection and gender, MCPyV seroprevalence was investigated in gender-stratified ES. To this purpose, the presence of IgG Abs against MCPyV was determined in sera from male (n=106) and female (n=120) ES and rates were compared. The prevalence of serum Abs reacting to MCPyV resulted as 67% (71/106) and 61% (73/120) in male and female groups, respectively, with no differences between the two gender groupings ($p>0.05$).

Table 7. Seroprevalence of IgG antibodies reacting with Merkel cell polyomavirus VP1 S and VP2 F peptides in healthy elderly subjects (ES).

			Number of positive samples		
Age yrs	**Number of samples**	**Male (%)**	**VP1 S**	**VP2 F**	**VP1 S+VP2 F**
66-70	40	52.5	28 (70)	26 (65)	25 (62.5)
71-75	46	50	33 (71.7)	39 (84.8)	33 (71.7)
76-80	57	56.1	39 (68.4)	38 (66.7)	37 (64.9)
81-85	47	36.2	32 (68.1)	32 (68.1)	30 (63.8)
85-100	36	36.1	21 (58.3)	26 (72.2)	19 (52.8)
Total	226	46.9	153 (67.7)	161 (71.2)	144 (63.7)

Serum samples (n=226) were from healthy elderly subjects. Statistical analyses were performed using the two-sided chi-square test. No statistical differences were detected among age-stratified groups (p>0.05) (170).

4.6.2 Serological profiles of IgG reacting to Merkel cell polyomavirus in healthy elderly subjects

Serological profiles of IgG Abs reactivity to S and F peptides, both for single peptides and in combination, were analysed. Immunological data were taken from the entire ES cohort (n=226), and results are reported as OD readings at λ 405 nm. The median (IQR) OD values for S peptide and F peptide in ES sera were 0.14 (0.10-0.18) and 0.25 (0.18-0.49), respectively, while for combined S and F peptides these resulted as 0.18 (0.13-0.30). The median (IQR) ODs for S peptide and F peptide, both for single mimotopes and in

combination, were then determined in age-stratified ES and values were compared. The median (IQR) OD values for S peptide resulted as 0.14 (0.1-0.17), 0.15 (0.11- 0.21), 0.13 (0.11-0.17), 0.13 (0.11-0.2) and 0.13 (0.1-0.19) in age-stratified ES groups aged 66-70, 71-75, 76-80, 81-85, 86-100 yrs old, respectively (Figure 13A). The median (IQR) OD values for F peptide resulted as 0.25 (0.17-0.53), 0.25 (0.18- 0.53), 0.24 (0.16-0.42), 0.29 (0.18-0.5) and 0.28 (0.2-0.46) in age-stratified ES groups aged 66-70, 71-75, 76-80, 81-85, 86-100 yrs old, respectively (Figure 13B). The median (IQR) ODs for combined S and F peptides resulted to be 0.17 (0.12-0.26), 0.19 (0.13- 0.37), 0.16 (0.12-0.26), 0.19 (0.12-0.31), 0.19 (0.12-0.32) in age-stratified ES groups aged 66-70, 71-75, 76-80, 81-85, 86-100 yrs old, respectively (Figure 13C). No statistically significant differences in OD values for peptides S and F, both for single peptides and in combination, were determined among age-stratified ES groups ($p>0.05$). Similarly, Spearman correlation analysis showed a lack of correlation between age and OD levels, for peptides S and F, in the entire cohort of ES ($p>0.05$) (data not shown).

The median (IQR) ODs for S peptide and F peptide, both for single peptides and in combination, were then determined in ES males (n=106) and females (n=120), whereas values were compared. The ODs for S and F peptides in the male group were 0.14 (0.11-0.2) and 0.24 (0.17-0.50), respectively, while these resulted as 0.13 (0.11-0.17) and 0.28 (0.18-0.49), respectively, in the female group (Figure 13D, E). Moreover, the median (IQR) ODs for combined S and F peptides were 0.19 (0.12-0.28) in males and 0.17 (0.13-0.30) in females (Figure 13F). No statistically significant differences in ODs for peptides S and F, both for single peptides and in combination, were determined between males and females ($p>0.05$).

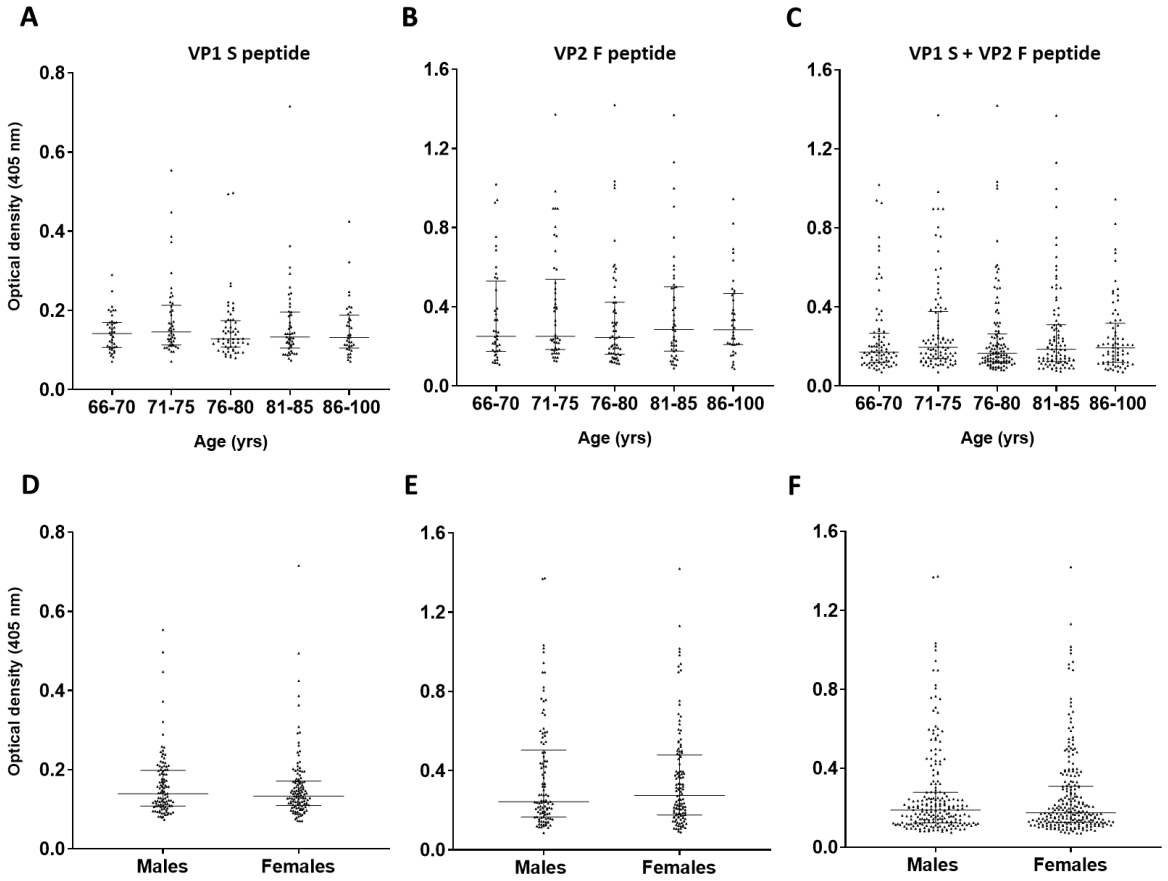


Figure 13. Serological profiles of serum antibody reactivity to Merkel cell polyomavirus VP1 S (A, D), VP2 F (B, E) peptides and for combined S and F peptides (C, F) in age- and gender-stratified healthy elderly subjects (ES). Immunologic data are from age- and gender-stratified ES and results are reported as optical density (OD) value readings at λ 405 nm for serum samples assayed in indirect ELISA. In the scatter dot plot, each dot represents the dispersion of ODs for each sample. The median is indicated by the line inside the scatter plot with the interquartile range (IQR) in age-stratified ES, i.e., 66-70 (n=40), 71-75 (n=46), 76-80 (n=57), 81-85 (n=47) and 86-100 (n=36) yrs and in gender-stratified ES, i.e., males (n=106) and females (n=120). No statistically significant differences in MCPyV seroreactivity were determined among age-/gender-stratified groups ($p>0.05$) (170).

4.7 Age-specific Merkel cell polyomavirus-seroprevalence and serological profile comparisons in the entire group of healthy individuals up to 100 years old

4.7.1 Age-specific Merkel cell polyomavirus-seroprevalence

In a second phase of the immunological investigation, the MCPyV-seroprevalence was compared among HC (0-20 yrs; n=344), HS (21-65 yrs; n=510) and ES (66-100 yrs; n=266) cohorts. Sera from HC, HS and ES which react to combined S and F peptides reached with an overall prevalence of 40.7% (140/344), 61.8% (315/510) and 63.7% (144/226), respectively. Notably, a lower IgG MCPyV-seroprevalence was found in HC cohort compared to both HS and ES cohorts ($p<0.0001$) (Figure 14). No statistical differences in MCPyV-seroprevalence were found when comparing ES and HS ($p>0.05$).

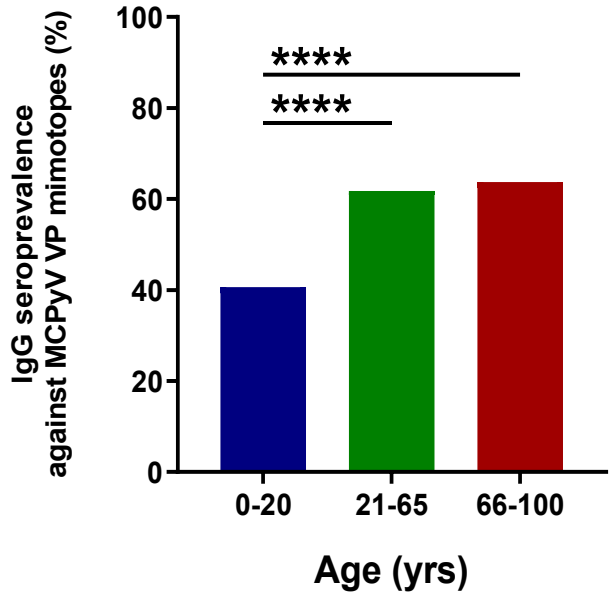


Figure 14. Seroprevalence of anti-Merkel cell polyomavirus IgG antibodies among healthy children (HC), healthy subjects (HS) and healthy elderly subjects (ES). Seroprevalences were compared in HC aged 0-20 yrs (n=344), HS aged 21-65 yrs (n=510) and ES aged 66-100 yrs (n=266). Statistical analyses were performed with two-sided chi-square test. The prevalence detected in HC group resulted significantly lower compared to HS and ES groups ($p<0.0001$).

MCPyV-seroprevalences were afterwards evaluated among age-stratified HC, HS, and ES. Groups were stratified in 5-yrs range cohorts and rates were compared. In the cohorts aged 0-5, 6-10, 11-15, 16-20, 21-25, 26-30, 31-35, 36-40, 41-45, 46-50, 51-55, 56-60, 61-65, 66-70, 71-80, 81-85 and 86-100 yrs, a prevalence pattern of combined S and F resulted as 13% (18/138), 52.3% (23/44), 60.5% (46/76), 61.6% (53/86), 47.5% (19/40), 63.5% (33/52), 57.6% (34/59), 55.7% (34/61), 60.5% (46/76), 69.2% (45/65), 72% (36/50), 63.6% (35/55), 63.5% (33/52) 62.5% (25/40), 71.7% (33/46), 64.9% (37/57), 63.8% (30/47) and 52.8% (19/36), respectively (Table 8). It should be underlined that a significantly lower seroprevalence was found in the 0-5 yrs cohort compared to the remaining age-stratified cohorts ($p<0.0001$) (Table 8). In addition, a lower MCPyV-seroprevalence was detected in the 21-25 yrs cohort compared to 46-50, 55-55 and 71-75 yrs cohorts ($p<0.05$) (Table 8). No statistical differences in MCPyV-seroprevalence were found among the remaining age-stratified groups ($p>0.05$) (Table 8).

Table 8. Seroprevalence of IgG antibodies reacting with Merkel cell polyomavirus VP1 S and VP2 F peptides in age-stratified heathy children (HC), healthy subjects (HS) and healthy elderly subjects (ES)

		Number of positive samples (%)
Age yrs	**Number of samples**	**VP1 S+VP2 F**
0-5	138	18 (13)*
6-10	44	23 (52.3)
11-15	76	46 (60.5)
16-20	86	53 (61.6)
21-25	40	19 (47.5)§
26-30	52	33 (63.5)
31-35	59	34 (57.6)
36-40	61	34 (55.7)
41-45	76	46 (60.5)
46-50	65	45 (69.2)
51-55	50	36 (72)
56-60	55	35 (63.6)
61-65	52	33 (63.5)
66-70	40	25 (62. 5)
71-75	46	33 (71.7)
76-80	57	37 (64.9)
81-85	47	30 (63.8)
86-100	36	19 (52.8)
Total	1080	599 (55.5)

Serum samples (n=1080) were from combined healthy children (HC), healthy subjects (HS), and healthy *elderly subjects (ES). Statistical analyses were performed using the two-sided chi-square test. *The different prevalence of MCPyV antibodies evaluated in 0-5 yrs age-stratified group was statistically significant compared to the remaining age-stratified cohorts ($p<0.0001$). §MCPyV-seroprevalence was different in the 21-25 yrs cohort compared to 46-50, 51-55 and 71-75 yrs cohorts ($p<0.05$).*

To assess any association between MCPyV infection and gender, the presence of IgG Abs against MCPyV were determined in HC (n=160), HS (n=204) and ES (n=106) males, as well as in HC (n=184), HS (n=306), and ES (n=120) and females and compared (Figure 15). HC males showed a lower MCPyV-seroprevalence of 37.5% (60/160) compared to 64.7% (132/204) and 67% (71/106), found in HS and ES males, respectively ($p<0.0001$) (Figure 15). Similarly, a significantly lower prevalence of 42.3% (80/184) was found in HC females compared to both HS, 59.8% (183/306), and ES, 61% (73/120), females (HC *vs* HS, $p<0.001$; HC *vs* ES, $p<0.05$, Figure 15).

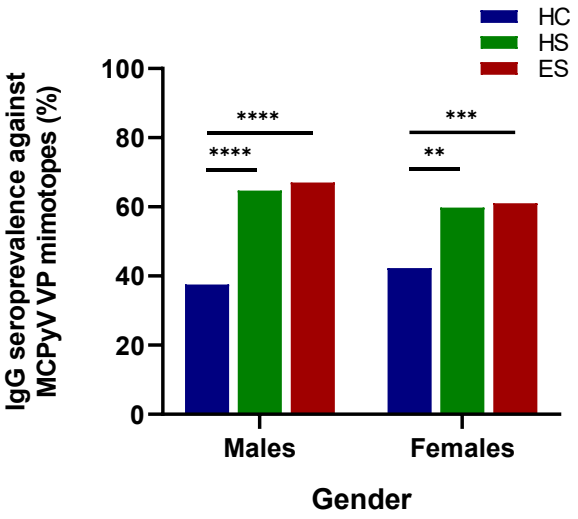


Figure 15. Seroprevalence of anti-Merkel cell polyomavirus IgG antibodies among gender- stratified healthy children (HC), healthy subjects (HS) and healthy elderly subjects (ES). Seroprevalences were compared in HC (n=160 males and n=184 females), HS (n=204 males and n=306 females) and ES (n=106 males and n=120 females. Statistical analyses were performed with two-sided chi-square test. The prevalence detected in HC males resulted significantly lower compared to HS and ES males ($p<0.0001$). HC females sowed lower prevalence compared to HS females ($p<0.01$) and ES females ($p<0.001$).

4.7.2 Age-specific serological profiles

MCPyV-serological profiles of both S and F peptides, alone and in combination, were then compared in HC (0-20 yrs; n=344), HS (21-65yrs; n=510) and ES (66-100 yrs; n=266) (Figure 16) cohorts. Notably, serological profile analysis indicated that F peptide ODs resulted lower in the HC group (median [IQR], 0.26, 0.17-0.38) than in HS (0.27, 0.2-0.41, $p<0.05$) (Figure 16B), while no differences were detected when HC were compared to ES (0.25, 0.18-0.49, $p>0.05$). Contrariwise, S peptide ODs were similar in HC (0.15, 0.1-0.22), HS (0.15, 0.11-0.20) and ES (0.14, 0.10-0.18) groups ($p>0.05$) (Figure 16A). No statistical differences for combined S and F peptides were found in HC group (0.2, 12-0.31) compared to both HS (0.2, 0.13-0.29) and ES (0.18, 0.13-0.30) ($p<0.05$) (Figure 16C).

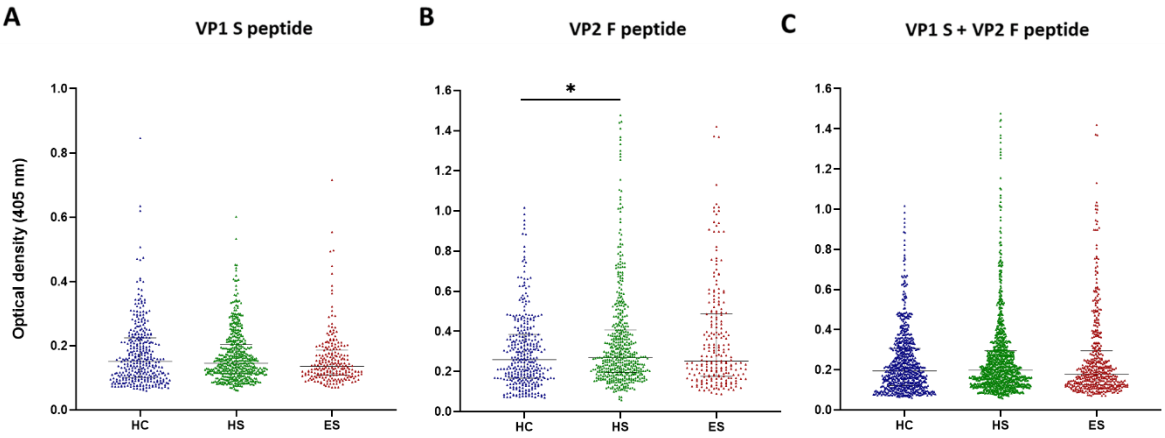


Figure 16. Serological profiles of serum antibody reactivity to Merkel cell polyomavirusVP1 S (A), VP2 F (B) peptides and for combined S and F peptides (C) evaluated in healthy children (HC) compared to healthy subjects (HS) and healthy elderly subjects (ES). Immunologic data are from HC (n=344), HS (n=510) and ES (n=226) results are reported as optical density (OD) value readings at λ 405 nm for sera analysed in indirect ELISA. In the scatter dot plot, each dot represents the dispersion of ODs for each sample. The median is indicated by the line inside the scatter plot with the interquartile range (IQR) in HC, HS, and ES cohorts. **(B)** *p<0.05.

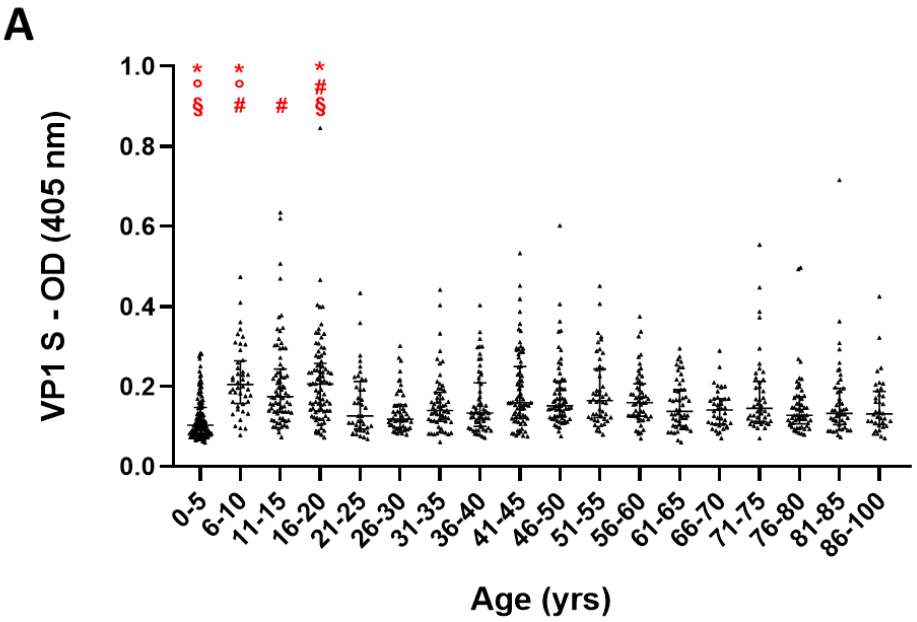
Serological profiles comparison among 5-yrs range age-stratified HC, HS and ES groups showed lower median (IQR) ODs for S peptide in 0-5 yrs (0.1, 0.08-0.15) cohort compared to 31-35 (0.14, 0.11-0.19), 36-40 (0.13, 0.1-0.21), 41-45 (0.16, 0.12-0.25), 46-50 (0.15, 0.12-0.21), 51-55 (0.16, 0.12-0.24), 56-60 (0.16, 0.13-0.21), 61-65 (0.14, 0.1-0.19), and 71-75 (0.15, 0.11-0.21) yrs cohorts ($p<0.05$, Figure 17A). In addition, lower median (IQR) ODs also for F peptide were detected in 0-5 yrs (0.16, 0.11-0.25) cohort compared to 26-30 (0.22, 0.19-0.35), 31-35 (0.23, 0.19-0.3), 36-40 (0.24, 0.17-0.44), 41-45 (0.32, 0.2-0.41), 46-50 (0.31, 0.22-0.5), 51-55 (0.32, 0.25-0.52), 56-60 (0.27, 0.2-0.44), 61-65 (0.27, 0.2-0.42), 66-70 (0.25, 0.17-0.53), 71-75 (0.25, 0.18-0.53), 76-80 (0.24, 0.16-0.42), 81-85 (0.29, 0.18-0.5) and 86-100 (0.28, 0.2-0.46) yrs cohorts ($p<0.05$, Figure 17B). Similarly, lower median (IQR) ODs for combined S and F peptides were detected in 0-5 yrs cohort (0.13, 0.09-0.19) when compared to 21-25 yrs (0.19, 0.11-0.26), 26-30 yrs (0.18, 0.12-0.26), 31-35 yrs (0.19, 0.13-0.26), 36-40 yrs (0.18, 0.12-0.28), 41-45 yrs (0.21, 0.15-0.35), 46-50 yrs (0.21, 0.15-0.35), 51-55 yrs (0.25, 0.16-0.33), 56-60 yrs (0.2, 0.14-0.29), 61-65 yrs (0.2, 0.12-0.27), 66-70 yrs (0.17, 0.12-0.26), 71-75 yrs (0.19, 0.13-0.37), 76-80 yrs (0.16, 0.12-0.26), 81-85 yrs (0.19, 0.12-0.31) and 86-100 yrs (0.19, 0.12-0.32) cohorts ($p<0.01$, Figure 17C).

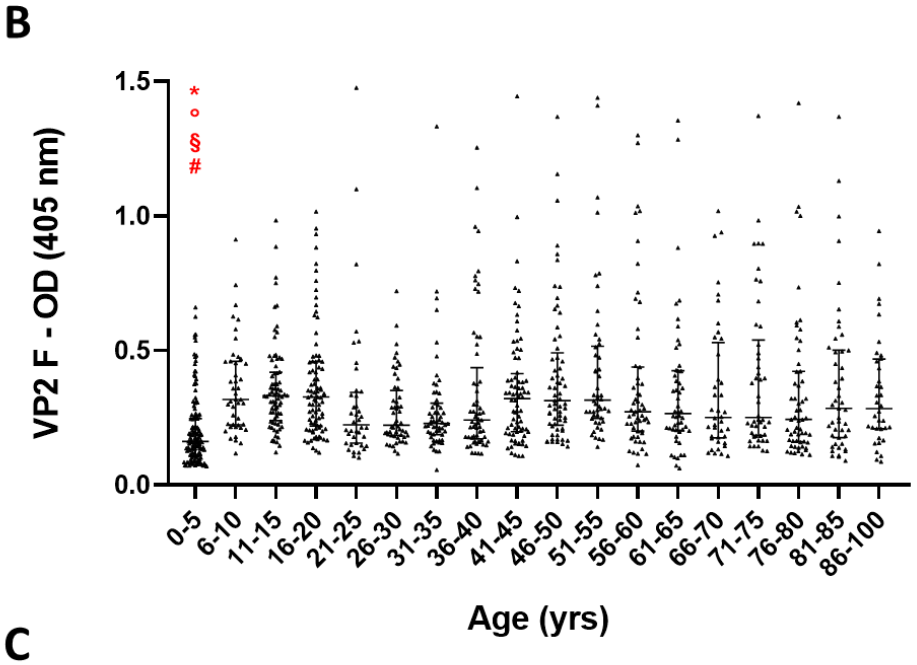
The 6-10 yrs cohort (0.21, 0.16-0.26) showed higher ODs for S peptide compared to the others aged 21-25 yrs (0.13, 0.1-0.21), 26-30 yrs (0.12, 0.1-0.15), 31-35 yrs, 36-40 yrs, 61-65 yrs, 66-70 yrs (0.14, 0.1-0.17), 76-80 yrs (0.13, 0.11-0.17), 81-85 yrs (0.13, 0.11-0.2) and 86-100 yrs (0.13, 0.1-0.19) ($p<0.05$, Figure 17A).

Likewise, higher median (IQR) OD levels for combined S and F peptides were found in 6-10 yrs cohort (0.23, 0.19-0.36) compared to 21-25yrs, 26-30 yrs, 31-35 yrs, 76-80 yrs ($p<0.05$, Figure 17C).

The cohort aged 11-15 yrs (0.18, 0.14-0.24) showed higher median (IQR) OD values for S peptide when compared to 26-30 yrs cohort ($p<0.01$). In addition, higher median (IQR) ODs for combined S and F peptides were detected in 11-15 yrs group (0.24, 0.16-0.35) when compared to 21-25 yrs, 26-30 yrs, 31-35 yrs and 76-80 yrs cohorts ($p<0.05$, Figure 17A).

Additionally, the cohort aged 16-20 yrs old (0.21, 0.14-0.26) showed higher (median (IQR) OD levels for S peptide compared to 21-25, 26-30, 36-40, 66-70 and 76-80 yrs groups ($p<0.05$, Figure 17A). Higher median (IQR) ODs for combined S and F peptides were detected in 16-20 yrs group (0.24, 0.16-0.35) when compared to 21-25, 26-30, 31-35, 36-40 and 76-80 ($p<0.05$, Figure 17C).


A
VP1 S - OD (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-100
Age (yrs)


B
VP2 F - OD (405 nm)
1.5
1.0
0.5
0.0
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-100
Age (yrs)

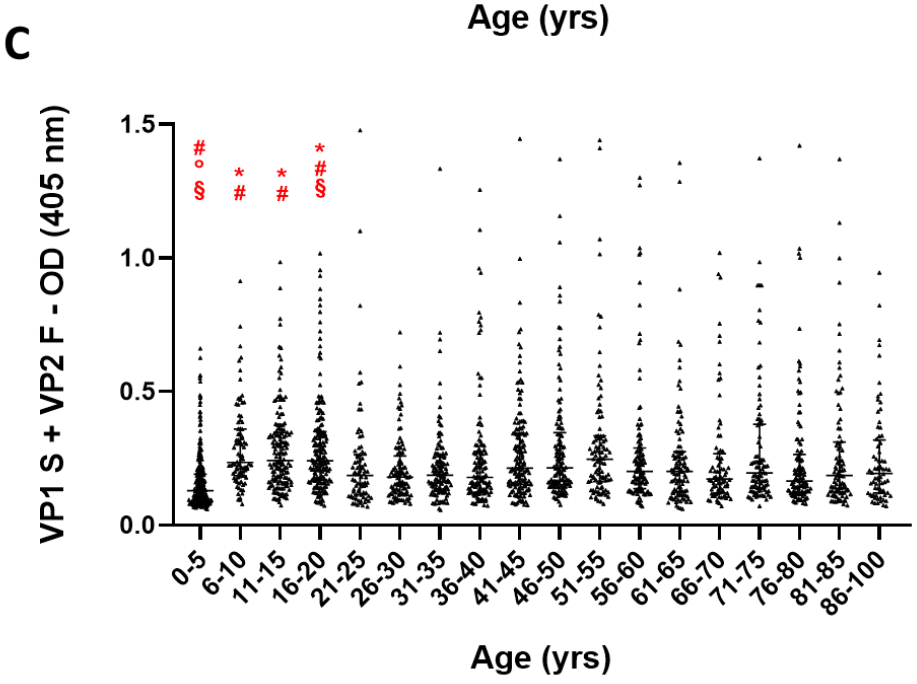

C
VP1 S + VP2 F - OD (405 nm)
1.5
1.0
0.5
0.0
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
51-55
56-60
61-65
66-70
71-75
76-80
81-85
86-100
Age (yrs)

Figure 17. Serological profiles of serum antibody reactivity to Merkel cell polyomavirus VP1 S (A), VP2 F (B) peptides and for combined S and F peptides (C) in age-stratified healthy children (HC), healthy subjects (HS) and healthy elderly subjects (ES). Immunologic data are from 5 yrs age-stratified HC, HS and ES and results are reported as optical density (OD) value readings at λ 405 nm for serum samples assayed in indirect ELISA. In the scatter dot plot, each dot represents the dispersion of ODs for each sample. The median is indicated by the line inside the scatter plot with the interquartile range (IQR). **(A)** °p<0.0001 0-5 *vs* 6-10, 11-15, 16-20, 41-45, 46-50, 51-55, 56-60; §p<0.001 *vs* 71-75; *p<0.05 *vs* 31-35, 36-40, 61-65; °p<0.0001 6-10 *vs* 26-30; #p<0.01 *vs* 76-80; *p<0.05 *vs* 21-25, 31-35, 36-40, 61-65, 66-70, 81-85, 86-100; #p<0.01 11-15 *vs* 26-30; §p<0.001 16-20 *vs* 26-30; #p<0.01 *vs* 76-80; *p<0.05 *vs* 21-25, 36-40, 66-70. **(B)** °p<0.0001 0-5 *vs* 6-10, 11-15, 16-20, 41-45, 46-50, 51-55, 56-60, 61-65, 71-75, 81-85; §p<0.001 *vs* 36-40, 86-100; #p<0.01 *vs* 66-70, 76-80; *p<0.05 *vs* 26-30, 31-35. **(C)** °p<0.0001 0-5 *vs* 6-10, 11-15, 16-20, 31-35, 36-40, 41-45, 46-50, 51-55, 56-60, 61-65, 71-75, 81-85; §p<0.001 *vs* 76-80, 86-100; #p<0.01 *vs* 21-25, 26-30, 66-70; #p<0.01 6-10 *vs* 26-30, 76-80; *p<0.05 *vs* 21-25, 31-35. #p<0.01 11-15 *vs* 26-30, 76-80; *p<0.05 *vs* 21-25, 31-35; §p<0.001 16-20 *vs* 26-30; #p<0.01 *vs* 76-80; *p<0.05 *vs* 21-25, 31-35, 36-40.

Furthermore, serological profiles for both individual S and F peptides and in combination were analysed and compared in HC (n=160), HS (n=204) and ES (n=106) males, as well as in HC (n=184), HS (n=306), and ES (n=120) females.

Median (IQR) ODs for F peptide resulted as lower in HC males (0.24, 0.16-0.36) than in HS males (0.29, 0.21-0.43, p<0.001) (Figure 18B), while no statistical differences in S peptide ODs were determined in HC and HS males (p>0.05) (Figure 18A). Furthermore, lower median (IQR) ODs for combined S and F peptides were detected in HC males (0.18, 0.12-0.3) when compared to HS males (0.21, 0.15-0.3, p<0.001) (Figure 18C). The median (IQR) ODs for both S and F peptide in HC females, were similar when compared to those in HS and ES females (p>0.05) (Figure 18D, E). No differences in median (IQR) ODs for combined S and F peptides were detected in HC, HS, and ES females (p>0.05, Figure 18F).

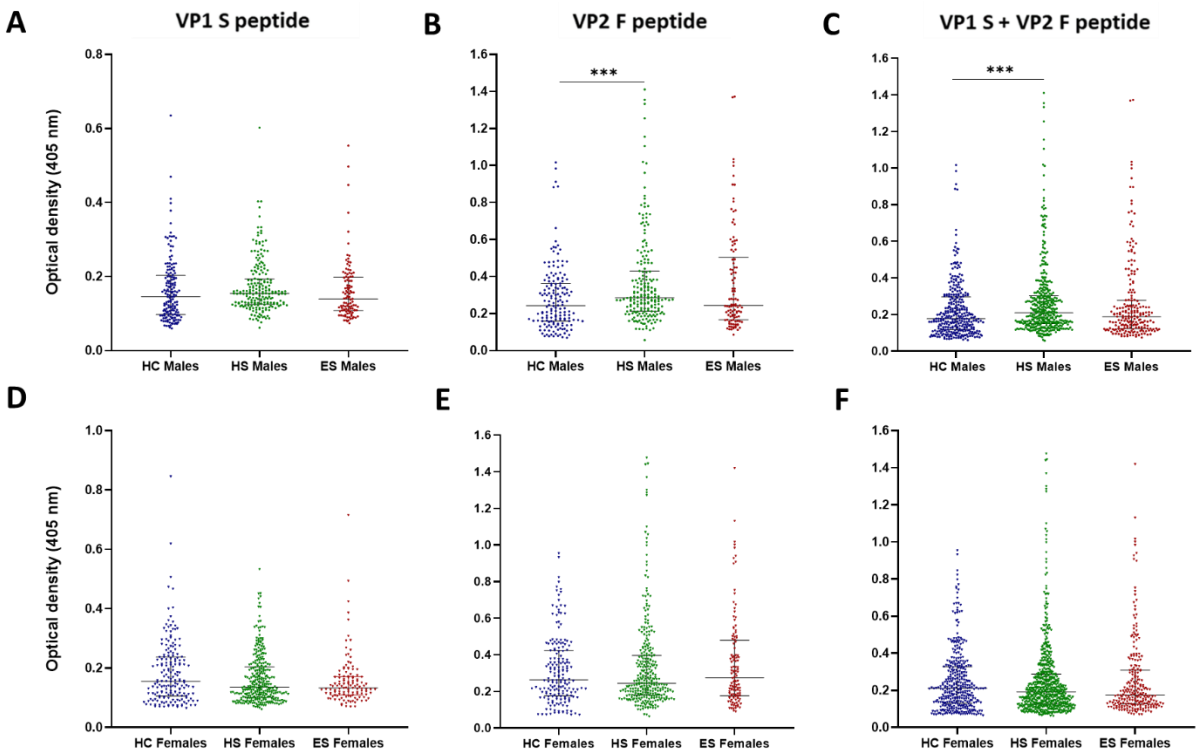


Figure 18. Serological profiles of serum antibody reactivity to Merkel cell polyomavirus VP1 S (A, D), VP2 F (B, E) peptides and for combined S and F peptides (C, F) evaluated in gender-stratified healthy children (HC) compared to gender-stratified healthy subjects (HS) and gender-stratified healthy elderly subjects (ES). Immunologic data are from gender-stratified HC (n=160 males and n=184 females), HS (n=204 males and n=306 females) and ES (n=106 males and n=120 females). Results are expressed as optical density (OD) value readings at λ 405 nm for serum samples assayed in indirect ELISA. In the scatter dot plot, each dot represents the dispersion of ODs for each sample. The median is indicated by the line inside the scatter plot with the interquartile range (IQR) for each group of subjects analysed. **(B)** and **(C)** ***p<0.001.

4.8 Preliminary evaluation of IC_{50s} of All-trans retinoic acid (ATRA) and Decitabine Merkel cell carcinoma cell lines

In the second phase of this study, preliminary dose findings experiments were conducted *in vitro* in MCC cells for investigating the effect of different concentrations of the antineoplastic compounds ATRA and decitabine on cell proliferation. Changes in proliferation were observed with WST-1 assay. Compounds were assayed at different concentration ranges in order to determine the specific IC_{50}-dose. In detail, dose findings assays conducted with decitabine provided the following concentrations 0.5 nM, 5 nM, 50 nM, 0.5 μM, 50 μM and 100 μM for five consecutive days (Figure 19); ATRA was titrated at 5 μM, 25 μM, 50 μM, 75 μM, 100 μM, 125 μM, 150 μM, while effects were determined after three days of treatment (Figure 19). Assays were carried out in MCCP cell lines MKL-1 and Peta and in MCCN cell lines MCC26, MCC13.

As shown in figure 19, MCC13 and Peta cell lines proved to be ATRA sensitive, with IC_{50s} ranging from 100 µM for MCC13 to 75 µM for Peta after 3 days of incubation. Intriguingly, ATRA induced only modest/null comparable anti-proliferative effects with concentrations values of 75-100 µM after 3 days of incubation in MCC26 cell line (Figure 19A).

Data showed that the highest effect of decitabine on cell proliferation inhibition was obtained at 0.5 µM in MCC13 and MCC26 on day five of treatment (Figure 19B). Similarly, MKL-1 cell line was found to be Decitabine sensitive with an IC_{50} being estimated at 50 nM on day five of treatment (Figure 19B).

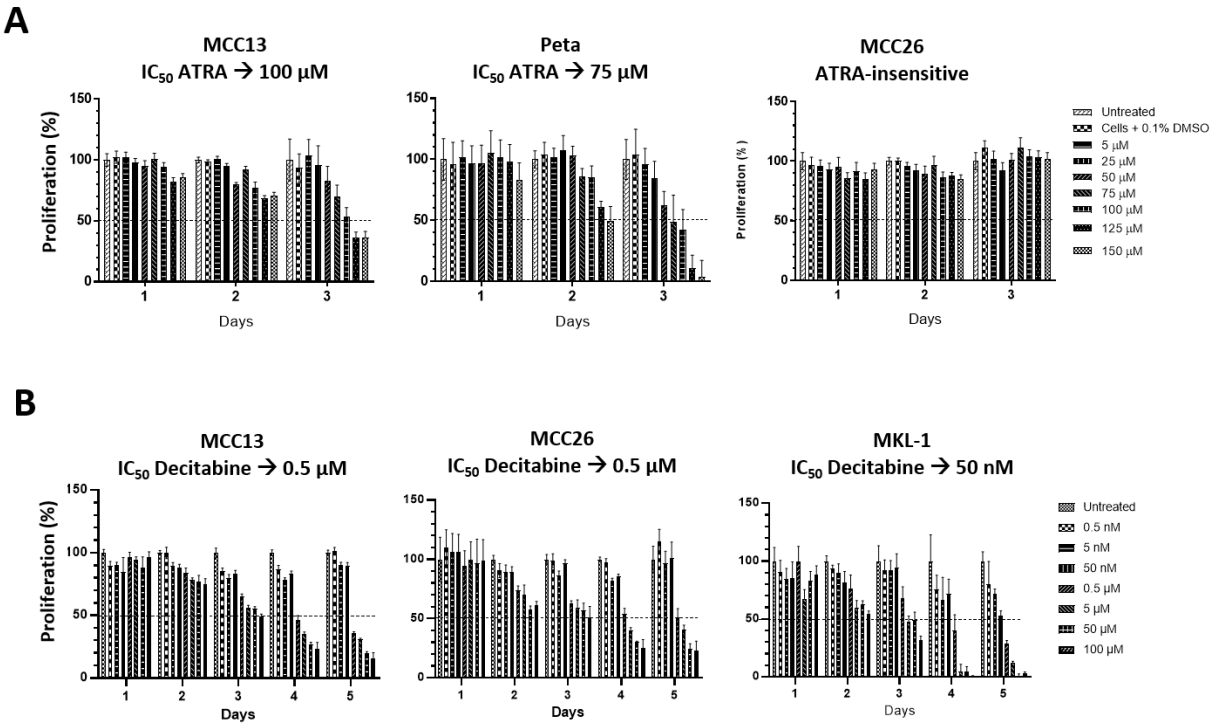


Figure 19. Dose-finding of Merkel cell polyomavirus (MPCyV)-positive Merkel cell carcinoma (MCCP) cell lines MKL-1 and Peta and MCPyV-negative (MCCN) MCC13 and MCC26 cell lines for All -trans retinoic acid (ATRA) and Decitabine. (A) Dose-finding for the ATRA IC_{50}-dose. Proliferation of MCC cells following treatment with increasing doses of ATRA was measured by WST-1 assay after 1, 2 and 3 days, normalized to vehicle control (0.015% DMSO). **(B)** Dose-finding for the IC_{50} concentration of Decitabine. Proliferation of MCC cell lines after treatment with increasing doses of Decitabine was measured by WST-1 assay after 1, 2, 3, 4, and 5 days, normalized to untreated cells. Pooled data of three independent measurements are shown, with three replicates in each experiment and cohort. Dashed line: IC_{50} value representing the minimal compound concentration required for a 50% inhibition of the cell proliferation.

5. Discussion

5. Discussion

5.1 Set-up of the new indirect ELISA assay and evaluation of the impact of Merkel cell polyomavirus infection in the general population

The present thesis describes the development and validation of a reliable indirect ELISA based on two synthetic peptides used as MCPyV VP mimotopes to detect circulating IgGs reacting with MCPyV VP antigens. The assay was set-up using 132 MCPyV-negative/-positive control sera, while its validation was performed on sera from different populations of healthy individuals with unknown MCPyV-serology comprising HC, HS an ES, for a total of 1080 sera, whose cumulative ages were ranging 0-100 yrs. Age- and gender-specific MCPyV-seroprevalence within and among cohorts were then determined. The need for more specific and rapid MCPyV-focused immunoassays prompted us to set-up and validate this new method.

In silico analyses were performed to assess the theoretical reliability of MCPyV VP1 S and VP2 F peptides as mimotopes/antigens. The S and F a.a. chains (i) are located within MCPyV VP1 and VP2 proteins, respectively, (ii) show shapes which are comparable to those of the native VPs, (iii) are exposed to the environment. In addition, S peptide is located on the VP1 BC surface loop (197). Both S/F positions may thus represent natural antigens establishing sites for immunoreactions. These computational data suggest that these peptides may be used for detecting IgGs to MCPyV by indirect ELISA, as they meet the requirement of mimotopes (198).

Indirect ELISA performance in detecting serum anti-MCPyV IgGs was then assessed on MCPyV-negative/-positive control sera. Controls were tested to compute a number of criteria which are commonly used in determining assay performances, including ELISA (177–182). Our assay proved to have adequate sensitivity (80%) and specificity (91%), with good PPV (94%) and NPV (71%), in detecting anti-MCPyV Abs, as described (177,199,200). Our method was also efficient, valid and accurate, as indicated by suitable rates ranging 84-86%, for the corresponding criteria (177). The expected/obtained results agreement was 86%, with a κ of 0.72, which is within the 0.61-0.80 range of substantial agreement (180,181,201). Acceptable J (0.71), LR+ (8.93) and LR- (0.22) values were also determined (178,179,202). In particular, a J towards 1 excludes the existence of false positives/negatives (178,202), while the higher (or lower) LR of a positive (or negative) test is, the more reliable is the result of the assay undergoing development (179). These trustworthy values cumulatively indicate a relevant correlation between expected and obtained results (199), thus underlining that the present immunoassay achieves suitable prerequisites for anti-viral IgGs detecting purposes (177,180,199,200). The performance of

each S and F peptide was assessed using ROC curve analysis, which is a key evaluating tool (183) and corresponding AUCs were calculated (181). AUCs of S (0.82) and F (0.74) peptides were both within the adequate 0.7-0.9 range (179,181), and higher than that of a worthless test (0.5, $p<0.001$) (203).

To evaluate assay precision in terms of repeatability and reproducibility, intra-/inter-assay CVs were measured (184). Marginal variations were found, as the variance between replicates reached the means over replicates and CVs were lower than 9%, thus below the acceptable 10-15% range (181,185,203). The assay, described in the present thesis, provides for the repeatable and reproducible detection of anti-MCPyV Abs and may be used for patient longitudinal monitoring, as abs could be recorded over time (204).

Then, the performance of the indirect ELISA was evaluated by (i) testing assay dilutional linearity (184,205–207), also known as accuracy, using sets of sera with known high, medium and low ODs, for MCPyV VP1 S and VP2 F peptides; (ii) comparing S and F peptide ODs. Both evaluations were performed on MCPyV-negative/positive control sera and in HC/ES sera. A remarkable correlation between ODs and sample dilutions was found, as linear regression analyses revealed an R^2 ranging 0.98-0.99 ($p<0.0001$) in two different evaluations being performed on MCPyV-positive control sera. This evidence indicates that our method is accurate (208,209). Spearman correlation analyses revealed that S and F peptide ODs were highly correlated when compared, with an r of 0.87 ($p<0.0001$) in MCPyV-negative/-positive control sera. Moreover, a good concordance between S and F peptide ODs was also observed in HC and ES groups, where r (s) of 0.8381 and 0.7991 ($p<0.0001$) were determined, respectively. These data indicate that not only is this assay robust in detecting anti-MCPyV IgGs with high accuracy, a key prerequisite for reliable ELISAs (169,208–212), but also S and F peptides can be exploited simultaneously, showing high concordance. In other words, the novel immunoassay presents a high reliability in detecting anti-MCPyV IgGs (169,177,181). In summary, these performance criteria cumulatively demonstrate that our indirect ELISA is reliable, sensitive, and specific in detecting IgG Abs to MCPyV.

Following the set-up steps, the assay was extended to sera from HC, HS and ES aged 0-20, 21-65 and 66-100 yrs, respectively, for a total of 1080 human sera with unknown MCPyV serology, while samples were tested for reactivity to MCPyV antigens. Indeed, one of the main objectives of this study was to determine whether sera from different healthy populations carried IgGs against MCPyV. Sera were considered positive when reacting with both S and F peptides, as previously performed (169,170).

The overall prevalence of IgGs to MCPyV in the HC cohort aged 0-20 yrs old was 40.7%. Previous data indicate that the seroepidemiology of MCPyV in children and young adolescent differs widely across studies, ranging from 3.4% to even 87.6% (9,57,62,64,65,73,95,213–215). The results of the present thesis are consistent with previous works reporting a prevalence ranging from 30% to 50% in young healthy individuals presenting an age range comparable to that of our study population (9,62,64,65,73,95,213–215). Although the immunoassay described herein does not allow our data and previous data to be adequately compared, variations in prevalence may reflect methodological disparities among assays (57,67). It should be underlined that previous MCPyV-based immunological methods mainly employ VLPs as antigens for immunoreactions, which are *in vitro*-generated recombinant MCPyV VPs (39,58,67,69,70,197,214). VLP-based assays might be susceptible to overestimation/cross-reaction, as VLPs shares common antigens with other PyVs (67,68,216), therefore potentially hampering the result (67,68). Previous high MCPyV serological rates have therefore been mainly obtained with ELISAs based on VLPs as antigens. As different PyVs show a certain degree of homology (67,68), data-invalidating cross-reactions cannot be excluded, when using VLPs. Our immunoassay differs from previous methods since it is based on two synthetic linear peptides mimicking portions of MCPyV VPs. Indeed, the S and F peptide a.a. chains used herein present a very marginal identity with corresponding VPs from other PyVs, as it has been demonstrated (169). Indeed, the S peptide a.a. chain is 100% concordant with that from VP native strains belonging to the five main MCPyV isolates, including North American, Japanese, European and Chinese isolates (23,194). The F peptide a.a. sequence is 100% identical with that to that of VPs from the North American and European isolates (23,194), while it presents 96% of similarity when compared with VPs from the Japanese and Chinese isolates and with AHW79949 and AWG42110 VP strains (23,194–196). Notably, sequence homologies for VPs from other PyVs were found to be remarkably low. This evidence supports the view that our assay is reliable in detecting circulating IgGs against MCPyV. In addition, VLP-based systems necessitate time consuming/expensive protocols (67,68), which might be susceptible to methodological flaws. Considering these aspects, our indirect ELISA exhibits high specificity in detecting anti-MCPyV IgGs, while cost/time-saving and a simple procedure are also provided (169).

Anti-MCPyV IgG seroprevalence was analysed herein in HC aged 0-20 years old. The lowest seroprevalence of IgG abs against MCPyV VP1/VP2 was observed in the 0-5 yrs age old cohort (13%) compared to the older age-stratified HC cohorts ($p<0.05$). Indeed, upon a primary strong increase occurring during the first years of life, MCPyV-serology seems to

remain quite stable throughout childhood starting from the 5th-6th yrs of life. These finding are consistent with previous studies suggesting an age-related increase in MCPyV-serology during 0-4 yrs peaking at about 10 yrs in MCPyV seroprevalence, then IgGs to MCPyV seems to stabilizes during prepuberal age (62,64,95,213,215). However, no age-related variations have also been reported in additional studies (9,65,73). In this context, further investigations are needed to clarify these inconsistencies across studies. Notably, MCPyV serological profile indicated that the younger group (0-5 yrs) had the lower OD levels compared to the older ones ($p<0.0001$). These results, consistent with those previously reported (213,215), may suggest that HC carry relatively low levels of anti-MCPyV IgGs that relatively continuously increase with age during childhood. Possibly, IgGs to MCPyV may increase in an age-dependent manner due to an early seroconversion, as previously described (9,64,213,215). In summary, the data reported in the present thesis alongside those reported previously provide the serological evidence of oncogenic MCPyV infection in the healthy paediatric population (9,57,62,64,65,73,95,213–215).

Analyses of gender-stratified HC showed no differences in terms of MCPyV seroprevalence between males and females, suggesting that MCPyV infection might be similarly distributed without gender-specific differences during childhood, as hypothesized (57,64,65,67,95,214,217). On the other hand, HC females showed herein higher anti-MCPyV IgG levels than age-matched males ($p<0.05$). This result may suggest the possible presence of differences in exposure or susceptibility to MCPyV infection during childhood. It should be noted that only one study found a gender difference with higher IgG levels in the male group compared to the female group in children aged 4-7 yrs (213). Therefore, additional studies on this issue would help us to explain these differences between males and females.

In the hypothesis that circulating anti-MCPyV IgGs might possibly increase in an age-dependent manner due to an early seroconversion (9,64,213,215), the overall IgM abs seroprevalence against MCPyV was thus evaluated in HC. The combination of MCPyV-positive sera, for both S and F peptides, resulted in an overall IgM prevalence of 29.7%; all age-stratified HC cohorts also presented comparable rates. The knowledge behind IgM abs against MCPyV during childhood is almost completely unknown (215). However, it should be underlined that our data are consistent with other seroepidemiological studies suggesting that primary infection with MCPyV most likely occurs during early childhood (213,215,218). Notably, quantitative data on anti-MCPyV IgM reactivity also indicated the presence of significant differences in OD values among age-stratified HC. Indeed, while the younger group aged 0-5 yrs had the lowest ODs compared to the remaining HC cohorts

($p<0.001$), 6-10 yrs and 11-15 yrs cohorts presented highest values compared to the older cohort aged 16-20 yrs ($p<0.01$). These results indicate, for the first time, that the paediatric population might be exposed to a primary MCPyV infection during the first years of life as reported for other pathogens (172,219,220). The IgM pattern described herein could thus explain the early immune response to MCPyV infection in childhood being previously hypothesised (57,61).

The anti-MCPyV IgM and IgG reactivity patterns were afterwards exanimated in parallel throughout the various HC ages. Both IgM and IgG had low ODs during the first years of life. Then, IgM ODs increased rapidly until peaking at about 10-12 yrs, while continuously decreasing until falling at around the 20^{th} yr of age. Conversely, IgG ODs remained constantly lower than those of IgM during the first years of life, while gradually increasing until peaking at the 20^{th} yr of age. These data indicate, for the first time, the presence of an opposite trend between anti-MCPyV IgG and IgM levels during the late childhood, with IgG levels being increased and IgM levels decreased in the older HC cohort. This is understandable as, after primary MCPyV infection which occur early in life, IgMs decrease due to seroconversion to IgGs during late childhood (63,65,73).

The serum IgM reactivity to MCPyV was also explored in males and females HC. A higher seroprevalence of anti-MCPyV IgMs in females compared to males was determined ($p<0.05$). This evidence was also confirmed by the IgM OD values, which were found to be higher in females ($p<0.01$). Gender-differences in IgM serology have also been reported for other pathogens in children (219). Notably, the present thesis is the first investigation reporting on a difference in IgM against MCPyV in terms of seroprevalence and ODs between HC males and females. In summary, the results of the present thesis suggest that MCPyV infection appears to be acquired early in life, possibly through familial and community transmission (95). Furthermore, this thesis highlights that MCPyV infection, like other PyV infections (9,65), is common in healthy children. Serological data obtained from HC showed that primary infection with MCPyV probably occurs in early childhood, perhaps following the disappearing of the specific maternal antibodies to MCPyV (9,95,198,213,215). The respiratory tract is a unique reservoir for MCPyV in children and it might be involved in the childhood transmission of the virus (73,221–223). For this reason, MCPyV transmission may occur when certain environmental conditions prevail, e.g., overcrowding and poor hygiene, and especially in areas where the background prevalence of MCPyV may be higher in the general population, such as community schools (95). However, the clinical implications of the presence of MCPyV in children remain to be elucidated.

The present immunoassay was afterwards extended on sera from HS aged 18-65 yrs old, with unknown MCPyV serology, while samples were studied for reactivity to MCPyV antigens. Indeed, another objective of this thesis was to determine whether sera from HS carried IgGs against MCPyV.

The overall seroprevalence of MCPyV in the entire cohort of HS was 62.9%. This finding indicates that MCPyV circulates widely in the adult human population, while its infection is asymptomatic. MCPyV seroepidemiology in the healthy adult population is highly variable across different studies, ranging 46-87% (9,39,65–67,69,214,216,217,224,57–64). As mentioned before in the present thesis, methodological disparities among studies might explain these variating ranges on MCPyV-serology. It should be noted that our results are in agreement with previous works reporting a prevalence ranging from 55% to 68%, in healthy individuals with a comparable age range to that of our study population (60,65,214,224). However, other reports have also described relatively high rates in adults, within 77-87% (57,67,95,217).

Analyses on age-stratified HS indicate that, although no statistical differences were found, MCPyV seroprevalence was slightly higher in the two older (41-50/51-65 yrs) groups, reaching 64.5 and 66.2%, respectively. MCPyV seroprevalence in healthy individuals has previously been found to increase with age (9,50,58,60–62,64,95,214), while no age-related variations have also been reported (57,67,214,217). A potential age-dependent seroprevalence pattern may imply that, despite the early seroconversion (65,73), MCPyV infection most likely occurs throughout life (60,64). Moreover, as MCPyV establishes a persistent infection, this kind of infection may also provide a permanent source of immune antigens resulting in a continuous/lifetime production of anti-viral IgGs (214). Notably, despite all HS having high ODs, the two older groups showed the highest levels ($p<0.05$). These findings, in agreement with those previously reported (57,62,64), may indicate that HS carry high levels of anti-MCPyV IgGs, which increase with age. IgGs may rise in an age-dependent manner as a result of the viral reactivation or reinfection with different strains and the following gradual production of antigenic MCPyV VPs, which is a frequent viral-related process during adulthood (225). Progressive viral reactivation during life may lead to viremia/immunoresponse (226), as MCPyV proteins are gradually exposed to the immune system.

Although a slightly higher number of HS sera from females was tested herein, gender-stratified HS proved no differences in IgG reactivity to MCPyV between males and females ($p>0.05$). This finding may suggest that MCPyV infection is equally distributed without disparities based on gender, as theorized (57,64,65,67,95,214,217). By contrast, in

this study, males showed higher ODs than females. This may imply that, although similar in term of prevalence, differences in exposure, or susceptibility to MCPyV infection may potentially exist in the HS studied herein. Higher anti-MCPyV IgG levels and/or seroprevalence rates have been described in healthy males compared to females (60,213). Additional investigations are needed to clarify this issue.

Detailed information on MCPyV serology in the healthy elderly population is still being debated (169). Herein, the impact of MCPyV infection in elderly individuals was determined on a set of ES sera aged from 66 to 100 years old with unknown MCPyV serology. MCPyV-seroprevalence and serological profile in ES according to age and gender were thus determined.

The overall prevalence, obtained by combining MCPyV-positive ES sera, for both peptides, resulted as 63.7%, a proportion higher to the 40.7% observed in HC cohort aged 0-20 yrs old, while similar to the 61.8% obtained in HS cohort aged 21-65 yrs old ($p<0.0001$); MCPyV seroprevalence was also higher in HS compared to HC ($p<0.0001$). These data cumulatively suggest that MCPyV seems to circulate asymptomatically not only in the young and adult population, but also in elders at a relatively high prevalence. Over the years, distinct prevalence figures across studies for individuals aged over 60 years old, with rates grouping from about 58-70% (9,50,60,61,214,224) to around 73-95% (57,58,64,65,67,95,217). Besides the abovementioned methodological differences among immunological studies, differences in geographical region/study populations could reflect these variations among studies (57,64). However, variating rates have also been reported across studies conducted in the same geographical region (9,50,57,61,62,64,69,95,217). Therefore, the currently available data do not allow robust conclusions to be reached about the existence of a MCPyV seroprevalence pattern depending on the geographical region. To clarify this question, multicentre studies based on large samples should be conducted.

No gender-related differences in terms of both MCPyV seroprevalence and ODs were determined in ES males and females ($p>0.05$). These data, in agreement with those reported previously (57,64,65,67,95,169), support the view that MCPyV infection might be homogenously distributed in elderly humans without gender-based disparity.

In summary, evaluating the impact of MCPyV infection in ES is of paramount importance for identifying individuals potentially at risk of MCC. Our indirect ELISA proved to be reliable in investigating circulating IgGs reacting to MCPyV VP mimotopes in ES sera. The results of the present thesis suggest that oncogenic MCPyV circulates in the elderly asymptomatically without variations according to age and gender, at a relatively high prevalence.

In order to gain knowledge on the impact of oncogenic MCPyV infection in the human healthy population, MCPyV-serology was afterwards examined in pooled HC, HS, and ES groups being stratified in 5-yrs range cohorts. A significantly lower MCPyV seroprevalence was found in the younger cohort aged 0-5 yrs (13%) compared to the remaining older cohorts (52.3-72%) ($p<0.0001$). These latter cohorts also presented similar overall rates were when compared ($p>0.05$); the lower rate exhibited by the 21-25 yrs cohort (47.5%) compared to the 46-50 (69.2%), 51-55 (72%) and 71-75 (71.7%) yrs cohorts was an exception ($p<0.05$). These data suggest that MCPyV seroprevalence increases with age during the early years of childhood, peaks at about 6-10 yrs, and remains nearly stable throughout the rest of life (57,64,214). In other words, after an early seroconversion during the early paediatric age, MCPyV seems to circulate asymptomatically across the prepuberal, adult and elderly populations at a relatively high prevalence, without substantial age-related variations during adulthood and senility.

Serological profiles of serum IgG reactivity to MCPyV evaluated in age-stratified HC, HS, and ES groups corroborate this hypothesis. When comparing OD values, the younger group, i.e., 0-5 yrs, showed the lowest ODs compared to the remaining older cohorts ($p<0.01$). Moreover, the higher OD values were found in cohorts aged 6-10 yrs, 11-15 yrs and 16-20 yrs compared to the remaining age-stratified cohorts; ODs constantly increased from the first years of life (0-5 yrs) to the last years of childhood (16-20 yrs), while decreased at 21-25 yrs and remained relatively constant throughout ages until senility (86-100 yrs). These immunological results indicate that, after a primary infection occurring during childhood, IgG levels against MCPyV remains almost continuously constant throughout life until senility. Although previous studies are characterised by fluctuating MCPyV seroprevalence rates (227), our results are consistent with previous studies reporting the lack of significant fluctuations with age (57,58,60,61,63,65,67,214). Upon early seroconversion during childhood, MCPyV establishes a lifelong asymptomatic infection in adults. In physiological conditions, this kind of infection may provide a long-lasting/lifetime source of viral antigens, which are permanently exposed to the host immune system, while continuous, consequent antigen stimulation is provoked (63,214). An alternative scenario provides that MCPyV antigens being gradually produced and exposed to the immune system during late childhood and adulthood, leading to a gradual increase in the immune response in an age-dependent manner (226), as described for other viruses (225). Indeed, as for other PyVs (73), the gradual production of anti-viral IgGs might be prompted by an increase in MCPyV replication levels or reinfections with different strains throughout life (60,64). Consistently, MCPyV seroreactivity has also been reported to increase with age (9,62,95,224).

Intriguingly, the older cohort, aged 86-100 yrs old, exhibited one of the relatively lowest MCPyV seroprevalence, i.e., 53%, that is approximately between 5-20% lower than the remaining HC/HS/ES cohorts aged below 85 yrs old. Moreover, the MCPyV seroprevalence in the 86-100 yrs old cohort was also almost identical to that of 6-10 yrs old cohort (52.3%). In addition, it was only 40% higher to that of the younger cohort aged 0-5 yrs old (13%); this difference resulted to be one of the lowest when the 0-5 yrs cohort was compared to the older cohorts. This result, in agreement with previous findings, suggests a possible decrease in MCPyV response during senility (64,65,217). Indeed, the immune system is prone to decline in old aged subjects (228), potentially leading to a reduced response to MCPyV. At the same time, as high MCPyV DNA amounts have been described in elders, a link between age and an increase in viral replication levels has also been theorized (229). Physiological immune senescence could therefore favour higher levels of MCPyV replication, as a minor response to MCPyV may occur (31,50,229,230), which in turn might lead to MCC carcinogenesis. This is understandable, as MCC mainly arises during advanced senility following MCPyV reactivation (34,231). The impairment of anti-viral/cancer immune surveillance in advanced age can therefore favour MCPyV-driven MCC carcinogenesis (232,233). Immunesenescence might be responsible for a decreased IgG antibody response to MCPyV, thereby potentially leading to an increase in MCPyV replication levels. In a few cases, alongside other risk factors, this phenomenon might prompt MCPyV-driven MCC onset, as described in elders aged over 60 years old (234). However, it should be noted that we did not find a substantial decrease in terms of anti-MCPyV IgG ODs in the 86-100 yrs old cohort compared to the younger cohorts. The reduced sample size available for statistical comparisons which hampered the result can be considered as an explanation.

In summary, the data in the present thesis, along with evidence reported previously (57,58,60,61,63,65,67,214), cumulatively suggest that, after seroconversion early in life (57,61), MCPyV infection seems to remain relatively stable throughout adulthood by evoking a physiological immune response, which might be followed by a decrease in MCPyV seroreactivity during advanced senility. The decrease in anti-viral/cancer immune surveillance might lead to higher MCPyV replication levels ultimately leading to MCC onset, despite occurring in certain critical circumstances, only (232,233).

A decrease in ODs was observed in HC males compared to HS males ($p<0.001$) (169). In addition, although no statistical differences, a decrease in ODs with age was also observed in males, but not in females, with low OD values detected in ES males compared to HS males. This evidence suggests that males might be more predisposed to develop

immune senescence than females, which in turn might possibly favour higher MCPyV replication levels. A gender-dimorphism in immune response impairment with age has been described as more pronounced in males than females (235). Indeed, although some evidences indicate a lack of gender differences (236), MCC seems to occur more frequently in males than females (237–240). No additional gender-related differences in terms of MCPyV-seroprevalence and serological profile were determined among HC, HS and ES groups ($p>0.05$).

Although the role of MCPyV in MCC is well-known (59), current MCPyV immunoassays are far from the clinical routine. These methods can necessitate several difficult tasks, as they require VLPs as antigens. This feature provides challenging/prolonged steps before ELISA protocol execution *per sé* (57,67,68,70). On these grounds, our new assay, using synthetic peptides, which is specific, rapid, and reliable in detecting Abs to MCPyV, may be used in routine clinical laboratory analysis. However, this strategy can be pursued by evaluating the antibody titre. The fine-tune of the circulating antibody titre against MCPyV with our immunological assay will be object of further studies. MCC-risk individuals/patients including blood donors/recipients, immunocompromised organ transplant recipients and oncologic/AIDS patients (85), as well as patients under iatrogenic immune compromission with biologics or with JAK inhibitors (23), can benefit from our assay. Indeed, monitoring of Abs against MCPyV is crucial for ensuring a good outcome for patients, by preventing MCC onset. In addition, since MCPyV LT/ST play an important role in MCC carcinogens (23,33,241), investigating the presence of serum IgGs against these viral oncoproteins in the healthy population, to identify MCC-risk individuals, might present clinical relevance. Our immunoassay can potentially be extended by employing novel synthetic linear peptides/mimotopes mimicking MCPyV LT/ST antigens, as previously performed for other PyVs (29). The simultaneous evaluation of IgGs against both MCPyV VPs and LT/ST antigens will allow a more comprehensive understanding of the impact of oncogenic MCPyV infection in the healthy population, in immunosuppressed patients and in MCC at risk patients. These experiments are feasible, and they could be part of next investigations.

The present study presents limitations. First, S and F peptides, used herein as mimotopes to detect circulating IgGs against MCPyV, were not tested for the detection of IgGs against MCPyV variants with mutations in the sequences corresponding to the used peptides. However, *in silico* analyses conducted previously to assess the theoretical reliability of the two peptides as mimotopes/antigens, indicated that both peptides were able to detect IgGs to a variety of known MCPyV strains with high concordance (169). Second,

a relatively small number of sera from HC, HS and ES has been tested. However, the sample size employed herein is statistically appropriate. Third, as MCPyV LT/sT plays a role in MCC onset/development (24,106), investigating the presence of serum IgGs against these viral oncoproteins in the healthy population, to identify MCC-risk individuals. Our ELISA method can potentially be extended by using different linear peptides/mimotopes mimicking portions of MCPyV LT/sT antigens, as performed previously (242). This methodological approach, which might allow a more comprehensive evaluation of the MCPyV infection in the healthy population, in immunosuppressed individuals and in MCC-risk patients, will be employed in further studies.

Developing methods to determine MCPyV-serology is needed in order to assess the impact of oncogenic MCPyV infection in humans. In this study, a reliable indirect ELISA for the detection of anti-MCPyV IgGs in sera from HC, HS and ES was successfully developed and validated. *In-silico* analyses indicated that linear peptides may specifically recognize IgGs generated against linear/conformational MCPyV VPs. Assay performance criteria, including sensitivity, accuracy, specificity, reliability, as well as other criteria, were thoroughly evaluated in MCPyV-positive/-negative controls and resulted as adequate. Our results therefore suggest that MCPyV infection, like other HPyV infections, is a relatively common event in healthy children, adults, and elders. Indeed, our data show that MCPyV infection has spread among healthy children, with a following relatively similar high prevalence in adults/elders and with a plausible decrease during senility. In summary, we may infer that oncogenic MCPyV is circulating asymptomatically at a relatively high prevalence in humans.

5.2 Preliminary evaluation of new therapeutic options in Merkel cell carcinoma treatment

The current therapies aimed at managing MCC-affected patients are limited (243). Novel therapeutic approaches are therefore urgently required (243). In the second phase of this thesis, as a preliminary approach, the antineoplastic effects of ATRA and the HMA decitabine were evaluated in MCC cell lines, with the aim of developing novel antitumor therapies applicable in the clinic. Specifically, the activities of the two drugs were assayed as a single compound *in vitro* in MCCP cell lines MKL-1 and Peta and in MCCN cell lines MCC26 and MCC13 to verify their anti-proliferative potential.

Dose-response experiments (141) were carried out to investigate, for the first time, the effect of different concentrations of ATRA on cell proliferation in MCC cell lines. Treatments with ATRA at various concentrations inhibited at different levels the MCC cell

proliferation, according to the cell line being considered. Indeed, results indicated that the IC_{50} value, i.e., the value representing the minimal ATRA concentration required for a 50% inhibition of the cell proliferation, was 100 μM and 75 μM in MCC13 and Peta, respectively. ATRA did not exhibit any anti-proliferative effect on MCC26. The tumour cell growth-inhibitory effect of retinoids, including ATRA, has been broadly documented. Retinoids, including ATRA, have been reported to present a strong anti-neoplastic effect both *in vitro* and *in vivo* in several tumour types, including breast, lung, ovarian, neuroblastoma, renal, pancreatic, liver, head and neck cancer (244,245). ATRA has also been reported to be effective in counteracting liquid tumour growth, such as acute myeloid leukaemia and acute promyelocytic leukaemia (140,246). Our data, in agreement with previous findings obtained in different tumour models, indicate that ATRA might induce anti-proliferative effects in MCC cells. Following ATRA administration, the inhibitory effect on cell proliferation might encompass the RARs/RXRs signalling pathway activation, (141). ATRA-driven cellular differentiation might prompt MCC cells to a reduced malignant phenotype, as previously described for other carcinomas (247). Since the cell growth inhibitory effect of ATRA was determined in the majority of MCC cell lines tested for the first time in the present thesis, our results suggest the possible involvement of RARs/RXRs activation in inhibiting MCC cell proliferation *in vitro*. On this ground, RARs/RXRs can be considered a pharmacological target to treat MCC affected patients. Hence, it will be of interest to further investigate the antineoplastic effect of ATRA in MCC cell lines by evaluating apoptosis and additional molecular parameters such as RARs/RXRs signalling pathway activation, which is known to favour cell differentiation (142). Since the lack of anti-proliferative effect for ATRA determined in MCC26, we cannot exclude the possibility that differences may exist between cell lines for RARs/RXRs pathway activation. Further studies are therefore needed to clarify the role of ATRA-induced RARs/RXRs activation in MCC cells. Moreover, exploring the RARs/RXRs pathway following ATRA treatment in order to identify novel combination therapeutic targets will be a valuable novel methodological approach for MCC therapy (140). These experiments are feasible, and they will be part of further investigations.

Epigenetically active inhibitor drugs directed at DNA methyltransferases (DNMTs), i.e., HMAs such as decitabine, have proven clinical efficacy in different haematological malignancies, as well as solid tumours (163,248–250). In the present thesis, we questioned whether MCC cell proliferation might be inhibited *in vitro* with the HMA decitabine. Dose-response experiments were therefore carried out in MCC13, MCC26 and MKL-1 cell lines. Notably, a strong effect of decitabine in inhibiting the cell proliferation was observed in all MCC cells investigated at variating concentrations according to the MCC histotype. In

particular, the IC_{50}-dose for cell growth-inhibitory effect was obtained at 0.5 μM in both MCC13 and MCC 26, while the HMA inhibited the proliferation of MKL-1 cells with a IC_{50}-dose of 50 nM. Epigenetics dysfunctions, such as improper DNA methylation, are involved in MCC aetiopathogenesis (132,156,251). The biological significance of DNA methylation is highlighted by the clinical efficacy of HMAs such as decitabine (163,164), which is currently in use for the treatment of various tumours (156,163). Furthermore, a recent study by Harms et al. conducted on MCC cells reported encouraging data with this HMA, resulting strongly effective against MCC *via* anti-proliferative activity, cell death, and increased immune recognition (156). Specifically, the antineoplastic effect of DAC has been analysed in a total of sixteen MCCP and MCCN cell lines in this study. Our results are in agreement with Harms et al., that reported an average IC_{50} of 252 nM for the HMA decitabine in MCCP and MCCN cell lines (156). A Phase II clinical trial for decitabine as salvage MCC therapy has been planned by the same group based upon their results (156). The preliminary results of the present thesis corroborate previous data demonstrating the strong anti-proliferative effect of decitabine in tumour cells, including MCC cells. These data, in agreement with those previously reported, therefore suggest that decitabine might be considered a novel antineoplastic compound for MCC therapy. However, the precise manner by which decitabine treatment exerts an inhibitory effect on MCC cell proliferation has been unleashed only partially (156). Further studies are required to explore the molecular mechanisms behind the anti-neoplastic effect of decitabine on MCC cells. For instance, methylome analyses might be useful for this purpose (156).

It should be recalled that the data obtained *in vitro* with ATRA and decitabine reported in the present thesis are preliminary and require further validation. Moreover, additional experiments such apoptosis evaluation, should be performed. If positive data will be obtained *in vitro*, *in vivo* experiments are required for assess the robustness of ATRA and decitabine as antineoplastic agents for MCC management. Consistently, further studies aimed in clarifying the antineoplastic role of ATRA and decitabine in MCC are in the planning phase.

In conclusion, both ATRA and decitabine demonstrated to be effective in reducing MCC cell proliferation *in vitro*. These important preliminary results might open the way to additional experimental approaches, such as combination treatments based on the simultaneous use of both antineoplastic agents. Indeed, combination therapies with different drugs are widely developed in preclinical research and clinical applications (252). These approaches are becoming part of routine evaluation in the set-up of novel antitumor treatments (252). MCC onset, progression and metastasis is often rapid (253). Since MCC is

considered an aggressive and deadly tumour, there is an urgent need to identify novel effective therapies. Combination multidrug therapies should therefore be designed (132), as being successfully demonstrated previously in treating other tumours (140,141,163,254–256). Further rigorous preclinical/clinical studies in this direction are required. For instance, MCC cell lines could be analysed to assess cell growth and invasiveness after drug treatment. Even though cell line screening provides faster results in a cost- and time-efficient manner, only cytotoxic agents can be identified. Notably, *in vitro* cytotoxicity is only one of the various effects that play a crucial role in the clinical efficacy of a candidate antitumor agent. Factors such as pharmacokinetics, toxicological evaluations and physiochemical properties are just as important as the pure efficacy of the anticancer agent. To this end, following the initial evaluation *in vitro*, the anticancer effect should also be meticulously evaluated *in vivo* with animal models. *In vivo* tumour models include either human tumour explants/xenografts or specially bred transgenic mice (257). *In vivo* tumour models have been shown to be more useful than *in vitro* assays for predicting clinical effects as multiple parameters such as pharmacokinetics, therapeutic index and efficacy of drug candidates are assessed simultaneously. Both *in vitro* and *in vivo* approaches should therefore be applied for evaluating novel antitumor agents for MCC therapy.

6. References

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7. Scientific contribution

During my PhD studies I have contributed to the publication of the following research articles:

1. **Mazziotta C**, Lanzillotti C, Pellielo G, Tognon M, Martini F and Rotondo JC. ***Significantly low levels of IgG antibodies against oncogenic Merkel cell polyomavirus in sera from females affected by spontaneous abortion***. Frontiers in Microbiology. doi: 10.3389/fmicb.2021.789991. IF= 5.640.

2. **Mazziotta C**, Lanzillotti C, Govoni M, Pellielo G, Mazzoni E, Tognon M, Martini F, Rotondo JC. ***Decreased prevalence of IgG antibodies reacting to viral protein mimotopes of oncogenic Merkelcell polyomavirus in sera from healthy elderly subjects***. Frontiers in Immunology, Viral Immunology. 2021. doi: 10.3389/fimmu.2021.738486. IF=6.429

3. Iaquinta MR, Torreggiani E, **Mazziotta C**, Ruffini A, Sprio S, Tampieri A, Tognon M, Martini F, Mazzoni E. ***In vitro osteoinductivity of hydroxylapatite scaffolds obtained with biomorphic transformation processes, assessed using human adipose stem cell cultures***. Int J Mol Sci. doi: 10.3390/ijms22137092. IF= 5.923

4. **Mazziotta C**, Lanzillotti C, Torreggiani E, Oton-Gonzalez L, Iaquinta MR, Mazzoni E, Gaboriaud P, Touzé A, Silvagni E, Govoni M, Martini F, Tognon M, Rotondo JC**.** ***Serum antibodies against the oncogenic Merkel Cell Polyomavirus detected by an innovative immunological assay with mimotopes in healthy subjects***. Frontiers in Immunology. **2021**. doi.org/10.3389/fimmu.2021.676627. Immunology 38/158 Q1. IF=7.561.

5. Oton-Gonzalez L, Rotondo JC, Cerritelli L, Malagutti N**,** Lanzillotti C, Bononi I, Ciorba A, BianchiniC, **Mazziotta C**, De Mattei M, Pelucchi S, Tognon M, Martini F. ***Association between HPV infectionand Killian polyp***. Infectious Agents and Cancer, Vol. 16, No. 1, pp: 1-9, **2021**. doi.org/10.1186/s13027-020-00342-3. IF=2.965.

6. Mazzoni E, **Mazziotta C**, Iaquinta MR, Lanzillotti C, D'Agostino A, Trevisiol L, Tognon M, Martini F. ***Enhanced Osteogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells by a Hybrid Hydroxylapatite/Collagen Scaffold***. Frontiers Cell and Developmental Biology,Vol. 8, No. 1, pp: 610570-610570, **2021**. doi:10.3389/fcell.2020.610570. IF=6.684.

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8. Rotondo JC, Oton-Gonzalez L, Selvatici R, Rizzo P, Pavasini R, Campo GC, Lanzillotti C, **MazziottaC**, De Mattei M, Tognon M, Martini F. ***SERPINA1 gene promoter is differentially methylated in peripheral blood mononuclear cells of pregnant women***. Frontiers in Cell and Developmental biology, Vol. 8, No. 1, **2020**. doi: 10.3389/fcell.2020.550543. IF=6.684.

9. Tognon M, Tagliapietra A, Magagnoli F, **Mazziotta C**, Oton-Gonzalez L, Lanzillotti C, Contini C, Vesce F, Rotondo JC, Martini F. ***Investigation on spontaneous abortion and Human Papillomavirus infection***. Vaccines, Vol. 8, No. 3, pp: 1-14, **2020**. doi: 10.3390/vaccines8030473. IF=4.422.

10. Mazzoni E, Pellegrinelli E, **Mazziotta C**, Lanzillotti C, Rotondo JC, Bononi I, Iaquinta MR, Manfrini M, Vesce F, Tognon M, Martini F. ***Mother-to-child transmission of oncogenic polyomaviruses BKPyV, JCPyV and SV40***. Journal of Infection, Vol. 80, No. 5, pp: 563-570, **2020**. doi: 10.1016/j.jinf.2020.02.006. IF=6.072.

During my PhD studies I have contributed to the publication of the following reviews:

11. **Mazziotta C,** Lanzillotti C, Gafà R, Touzé A, Durand MA, Martini F, Rotondo JC. ***The role of histone post-translational modifications in Merkel cell carcinoma.*** Front. Oncol. **2022**. doi: 10.3389/fonc.2022.832047. IF= 6.244

12. Oton-Gonzalez L, **Mazziotta C,** Iaquinta MR, Mazzoni E, Nocini R, Trevisiol L, D'Agostino A, Tognon M, Rotondo JC, Martini F. ***Genetics and Epigenetics of Bone Remodeling and Metabolic Bone Diseases.*** International journal of Molecular Sciences. **2022**. doi: 10.3390/ijms23031500. IF= 6.684.

13. Rotondo JC, **Mazziotta C**, Lanzillotti C, Tognon M, Martini F. ***Epigenetic dysregulations in Merkel cell polyomavirus-driven Merkel cell carcinoma***. International journal of Molecular Sciences, **2021**.doi: 10.3390/ijms222111464. IF= 5.923.

14. **Mazziotta C**, Rotondo JC, Lanzillotti C, Campione G, Martini F and Tognon M. **Cancer biology andmolecular genetics of A3 adenosine receptor.** Oncogene. **2021**. doi: 10.1038/s41388-021-02090-z. IF= 9.867

15. Rotondo JC, Martini F, Maritati M, **Mazziotta C**, Di Mauro G, Lanzillotti C, Barp N, Gallerani A, Tognon M, Contini C. ***SARS-CoV-2 infection: new molecular, phylogenetic, and pathogenetic insights. Safety of current vaccines and the potential risk of variants***. Viruses **2021**, 13(9). doi.org/10.3390/v13091687. IF=5.048

16. Rotondo JC, Lanzillotti C, **Mazziotta C**, Tognon M, Martini F. ***Epigenetics of male infertility: the role of DNA methylation***. Frontiers in Cell and Developmental biology. **2021**. doi: 10.3389/fcell.2021.689624. IF= 6.684.

17. Iaquinta MR*, Lanzillotti C*, **Mazziotta C***, Bononi I, Frontini F, Mazzoni E, Oton-Gonzalez L, Rotondo JC, Torreggiani E, Tognon M, Martini F. ***The role of microRNAs in the osteogenic and chondrogenic differentiation of mesenchymal stem cells and bone pathologies***. Theranostics, Vol.11, No. 13, **2021**. doi:10.7150/thno.55664. IF= 11.556. ***Co-first authors.**

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19. **Mazziotta C***, Lanzillotti C*, Iaquinta MR*, Taraballi F, Torreggiani E, Rotondo JC, Oton-GonzalezL, Mazzoni E, Frontini F, Bononi I, De Mattei M, Tognon M, Martini F. ***MicroRNAs regulate signaling pathways in osteogenic differentiation of mesenchymal stem cells***. International journalof Molecular Sciences, Vol. 22, No. 5, **2021**. doi: 10.3390/ijms22052362. IF= 5.923. ***Co-first authors.**

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21. Iaquinta MR, Mazzoni E, Bononi I, Rotondo JC, **Mazziotta C**, Montesi M, Sprio S, Tampieri A, Tognon M, Martini F**.** ***Adult Stem Cells for Bone Regeneration and Repair.*** Front Cell Dev Biol. **2019** Nov 12;7:268. doi: 10.3389/fcell.2019.00268. IF=6.684.