Role of human polyomaviruses in lymphoproliferative disorders and bladder cancer

Claudia Robles Hellín

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THESIS DIRECTORS

Dra. Silvia de Sanjosé

Cancer Epidemiology Research Programme , Unit of Infections and Cancer, Catalan Institute of Oncology (ICO), L'Hospitalet de Llobregat, Spain

Dr. Manolis Kogevinas

Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain

DEPARTAMENT OF EXPERIMENTAL AND HEALTH SCIENCES

upf. Universitat Pompeu Fabra Barcelona

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Sovint es cita en literatura el primer paràgraf de "A tale of two cities" de Charles Dickens: "*It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, (...).*". A grans trets, crec que aquestes primeres línies resumeixen els últims (gairebé) 4 anys de aprenentatge, un període ple d'adjectius oposats.

Epidemiològicament parlant però, caldria afegir que també ha estat un període d'interaccions (personals), confusions (mentals) i ajustaments (conceptuals). Així que perdoneu-me per endavant si algú no se sent identificat entre els següents agraïments:

En primer lloc, vull agrair a Silvia de Sanjosé i Manolis Kogevinas, que m'han guiat durant tot el procés i sense les quals definitivament aquesta tesi no hagués estat possible. No podria haver somiat amb una combinació millor d'entusiasme, paciència (molta paciència), pragmatisme, coneixements i sentit de l'humor. (Encara ara em pregunto com els vaig enredar per a què m'acceptessin com a estudiant...)

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Un milió de gràcies a tots!!

ABSTRACT

Polyomaviruses have been suspected to cause cancer in humans although, to date, Merkel cell polyomavirus (MCPyV) is the only member of this family that is a proven human carcinogen. This thesis explores the association of up to nine polyomaviruses with 468 lymphoproliferative disorders, 1135 bladder cancer and 359 chronic lymphocytic leukemia (CLL) subjects using three different case-control studies in Spain. Viral exposure was measured as seroreactivity against these viruses by virus-like-particles enzyme linked immunosorbent assay and fluorescent bead-based multiplex serology technology. In lymphomas, higher MCPyV seroprevalences were observed in most of lymphoma subtypes but only diffuse large b-cell lymphoma (DLBCL) showed a significant six-times higher MCPyV seroprevalence (OR=6.10; 95%CI=1.88-19.75) than controls, but no risk variation was observed with seroreactivity levels. Bladder cancer cases showed significant higher seroreactivities against BKPyV (OR= 1.37; 95%CI=1.04-1.80) and MCPyV (OR=1.48; 95%CI=1.16-1.88). In CLL, lower seroprevalences for the nine polyomaviruses tested were obtained (OR range=0.21-0.70).

The serological patterns obtained in DLBCL and bladder cancer are not fully consistent with a carcinogenic role and cannot exclude reactive patterns associated with the disease. Confirmation by means of prospective and molecular studies is required to adequately interpret the associations obtained. Our results in CLL are inconclusive for carcinogenesis due to the strong low seroreactivity observed. It is however unlikely that any of polyomavirus explored could play a role in CLL

RESUM

Fa temps que es sospita que els *Polyomaviruses* poden causar càncer en humans tot i que, fins al moment, el poliomavirus de cèl·lules de Merkel (MCPyV) es l'únic membre d'aquesta família que es carcinogènic demostrat en humans. Aquesta tesi explora les associacions de fins a nou poliomavirus en un total de casos de 468 trastorns limfoproliferatius, 1135 càncers de bufeta i 359 leucèmies limfocítica crònica (LLC) mitjançant l'ús de tres estudis cas-control diferents a Espanya. La exposició viral es va mesurar mitjançant *enzyme linked immunosorbent assay* amb *virus-like-particles* i tecnologia múltiplex de serologia fluorescent basada en *beads*. En limfomes, es van observar elevades seroprevalences de MCPyV en la majoria de subtipus, però nomes el limfoma difús de cèl·lules b grans (LDCBG) va mostrar una seroprevalença de MCPyV significativament 6-cops mes alta (OR=6.10; 95%CI=1.88-19.75) que en controls, tot i que no es van trobar diferencies en risc amb seroreactivitat. Els casos de càncer de bufeta van mostrar elevades seroreactivitats envers BKPyV (OR= 1.37; 95%CI=1.04-1.80) i MCPyV (OR=1.48; 95%CI=1.16-1.88). En LLC, es van observar seroprevalences més baixes per als 9 poliomavirus mesurats (OR rang=0.21-0.70).

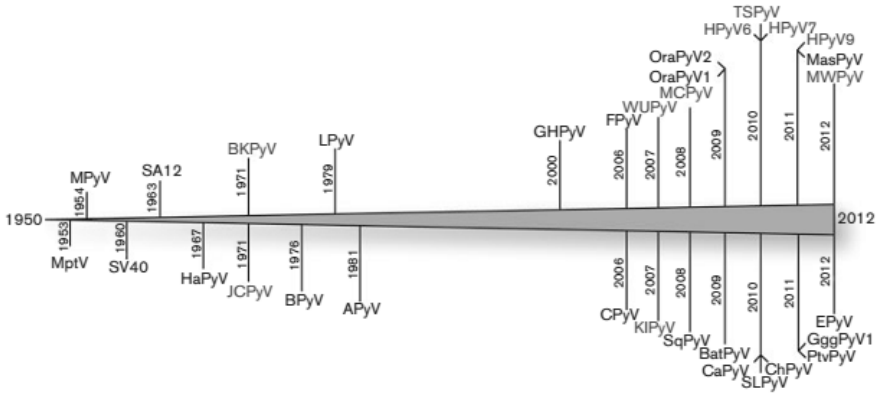
Els patrons serològics obtinguts en LDCBG y en càncer de bufeta no acaben de concordar amb un rol carcinogènic i patrons reactius degut a la malaltia no es poden descartar. Es necessita confirmació mitjançant estudis prospectius i moleculars per a la correcta interpretació de les associacions obtingudes. Els nostres resultats en LLC son inconclusius per a carcinogènesi donades les baixes seroreactivitats observades. Tot i així, es poc probable que algun dels poliomavirus estudiats jugui algun paper en LLC.

PREFACE

In 1953, the first polyomavirus was identified and its ability to cause cancer in mice was observed. Seven years later, SV40 was isolated from monkey cells and also able to induce cancer in animals. The potential carcinogenicity of SV40 in humans has been largely studied in the last decades because of SV40-contaminated polio vaccines, worldwide distributed and administered in the late 50s. However, solid data indicate that SV40 cannot be classified as carcinogenic to humans.

In 1971, the first polyomaviruses confirmed to infect humans were discovered; BKPyV and JCPyV. Both viruses have been associated with human diseases (polyomavirus-associated nephropathy for BKPyV and progressive multifocal leukoencephalopathy for JCPyV), but although they can induce cancer in animals, no carcinogenic association has been established in humans.

Since then, more than 35 years have been necessary to identify new human polyomaviruses. The implementation of new technologies based on molecular biology, such as the detection and sequencing of viral DNA rather than the virus identification via microscope or via isolation and culture in adequate media, have resulted in the identification of ten new human polyomavirus species.



Timeline of polyomaviruses isolation dates by Feltkamp et al.[39], updated to October 2012.

Among these newly identified polyomaviruses, a major breakthrough has been the isolation of Merkel cell polyomavirus in over 80% of Merkel cell carcinomas in 2008. The carcinogenic evidence provided in the last years on this association has been so consistent across laboratories, that in an unprecedented short period of time (only 4 years), the IARC concluded in 2012 on its *probable* human carcinogenesis.

Regarding the newly identified polyomaviruses other than MCPyV, although *in vitro* carcinogenicity has not been studied, they all contain preserved molecular characteristics that could lead to cellular transforming ability.

Little is known about how these viruses are transmitted but an oro-fecal transmission is the predominant theory for most of them. Furthermore, the high seroprevalences (>60%) of these viruses in humans and its common presence in our environment (river and residual waters), suggest that these viruses are easily acquired probably during childhood and coexist with humans without any further consequence. However, under specific circumstances not fully elucidated (immunosuppression, viral mutations, host characteristics…), these viruses can be reactivated, proliferate and lead to disease.

In this thesis, potential associations of polyomaviruses and human cancer are explored by means of three large multicentric case-control studies in which polyomaviruses seroreactivities have been measured. The work provides unique information for Spanish subjects with cancer and compares it with that observed among healthy subjects. Additionally, relevant aspects of the epidemiology of these viruses and their relationship with human disease have been studied.

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1. INTRODUCTION

1.1 Tumor virology

a) Viral associations to cancer

To date, the International Agency for Research in Cancer (IARC), has assessed and classified eight viruses (see Table 1) as carcinogenic to humans based on *sufficient* evidence in humans to increase the incidence of malignant neoplasms, reduce their latency or increase their severity or multiplicity[1,2].

Table 1. Viruses associated with cancer sites

Cancer sites	*Viral agents associated*
Liver	Hepatitis B virus (HBV) Hepatitis C virus (HCV)
Cervix uteri	Human papillomavirus (HPV) Human immunodeficiency virus (HIV)
Anogenital	Human papillomavirus Human immunodeficiency virus
Nasopharynx	Epstein-Barr virus (EBV)
Oropharynx	Human papillomavirus
Kaposi's sarcoma	Kaposi's sarcoma herpesvirus (KSHV) Human immunodeficiency virus
Non-Hodgkin lymphoma (NHL)	Epstein-Barr virus Hepatitis C virus Human T-lymphotropic virus type 1 Human immunodeficiency virus
Hodgkin lymphoma (HL)	Epstein-Barr virus Human immunodeficiency virus
Skin	Merkel cell polyomavirus (MCPyV)

b) Establishment of an infectious etiology in cancer

The etiological association between a viral agent and a given malignant long evolving process such as cancer is not straightforward. When exploring the etiological association of viral

agents and cancer, the following issues need to be taken into account; (i) the long incubation period between initial infection of putative virus and cancer development, which can range between 15 and 40 years, (ii) the ubiquitous presence of candidate virus but low incidence of cancer in overall population, (iii) the difficulty to establish the initial infection time point because the infection is often subclinical, (iv) the role of cofactors (such as host-related, environment and virus-related factors) that may be responsible for viral activation and subsequent carcinogenicity, (v) the complexity and multistage processes involved in cancer development and (vi) the inability of known human carcinogenic viruses to reproduce the human cancer under study in experimental animals[3,4].

The IARC, by consensus of a group of experts and specialists in the hazard being assessed, categorizes it as carcinogenic, independently of its nature (e.g. chemical, biological,…) and of its underlying mechanisms of carcinogenicity. To reach a conclusion, data on exposure, epidemiological studies in humans, studies in animals and other relevant data is summarized and used to establish the strength of the evidence. When the hazard under evaluation is an infection, data exposure will contain the mode of replication, life cycle, target cells, persistence, latency, host response and related clinical diseases other than cancer[1].

To date, infections have been broadly categorized into two main mechanisms of carcinogenesis; direct and indirect. Most viruses are *direct* carcinogens because of their ability to introduce into the host cell their viral oncogenes, or modify them after integration, causing the cell transformation. According to the IARC, under the direct mechanism, the viral genome or part of it is usually detected in a large part of the malignant cells, the virus can immortalize infected cells *in vitro* and the virus expresses several oncogenes that interact with cellular proteins and cause disruption of cell cycle checkpoints, apoptosis inhibition and cell immortalization[1]. On the other hand, other viruses may induce carcinogenesis through an *indirect* mechanism mainly led by a persistent tissue inflammation. A clear non-viral example is the one observed for *Helicobacter pylori* and stomach cancer[5]. It is believed that some hepatitis viruses could also act through a similar indirect mechanism by an increased production of pro-inflammatory molecules, which (i) enhance immune system deregulation, (ii) promote angiogenesis, and

(iii) lead to production of reactive oxygen species, which have mutagenic effects. Another *indirect* path to carcinogenesis is the one observed for HIV, which infects vital cells of the human immune system, leading to a poor immune response that allows direct carcinogenic agents to escape from immune control. A well know example is the strong link between HIV and lymphomas mediated by an immunosurveillance loss of EBV latent infections[6].

Epidemiology has played and will be playing an important role both in the discovery and in the causal association between human cancer and infections, especially for those with an indirect carcinogenic mechanism. Similar distributions of regional cancer and infection prevalence, increased incidence of cancer in immunosuppressed populations, regional clustering of cancer cases and cancer incidence related to climatic conditions have allowed the targeting of specific cancer sites suspected to have an infectious etiology[6]. On the other hand, relevant contributions of epidemiological studies in the establishment of causality of tumor virus in the past include the higher EBV seroprevalence observed in Burkitt lymphoma subjects when compared to controls[7], the increased incidence of hepatocellular cancer among HBV carriers[8] and the recently decreased incidence of hepatocellular cancer observed among young adults vaccinated against HBV[9].

c) Burden of cancer associated with infections and its potential prevention

In 2012[10], the burden of cancer associated with infections was estimated based on the associations listed in the IARC monograph as carcinogenic[1], which includes the associations listed in Table 1 and those for non-viral infections. Cancer sites with a non-viral infectious etiology include stomach (*Helicobacter pylori*), liver (*Opistarchis viverrini* and *Chlonorchis sinensis*), bladder cancer (*Schistosoma haematobium*) and non-Hodgkin lymphoma (*H. pylori*).

Cancer incidence data was obtained from GLOBOCAN 2008[11] and cancer registry data[12]. Attributable fraction of cancer to infections was estimated using the relative risk estimates of the infection, detailed in the monograph or estimated via meta-analyses, and the prevalence of infection in the population at risk.

Among the 12.7 million incident cancer cases detected worldwide in 2008, 16.1% (around 2 million cases) were estimated to be caused by infections. This attributable fraction ranged from 3.3% in Australia and New Zealand to 32.7% in sub-Saharian Africa, with an 80% of all the cancers linked to infections having occurred in developing countries. The burden estimation, however, followed conservative criteria; (i) the estimates did not include other potential associations such as EBV in gastric cancer, HBV in NHL, HPV in oral cavity neither MCPyV in Merkel cell carcinoma (not yet confirmed at the moment of analysis) and (ii) for some relative risks, although these infections are suspected to have a higher impact in the cancer burden, lower conservative values were used. Therefore, the obtained attributable fraction may be underestimating the real one.

More than 90% of the cancer burden related to infections was attributed to only hepatitis viruses, HPV and *H. pylori* infections. Since infections are a potentially modifiable external risk factor, prevention policies and strategies can be implemented. Prevention strategies include (i) control of contaminated blood products, medical instruments and tattooing instruments as well as needle exchange programs (HBV, HCV and HIV), (ii) enhance safe sex practices (HBV and HIV), (iii) early detection of precancerous or cancerous lesions through screening programs (HPV-related lesions), (iv) treatment of infection (use of antibiotic drugs for *H. pylori*) and (v) use of prophylaxis vaccines (HBV and HPV)[13,14].

d) Future perspectives in tumor virology

A large number of cancer sites are not fully explained by the known risk factors associated with them. Among these, some of them show increased incidences in immunosuppressed population, such as subjects with HIV/AIDS or organ-transplanted subjects[15,16] (see Figure 1). These increased incidences are likely to be related to infections, already discovered or yet to be isolated. Therefore, cancer sites listed in the figure (kidney, melanoma, bladder, thyroid, brain and testis cancer as well as multiple myeloma and leukemia) are prominent cancer site candidates. Additionally, not all AIDS-related cancers, such as NHL, are fully attributed to its associated

infectious risk factor, and therefore remain as cancer sites candidates to other infections.

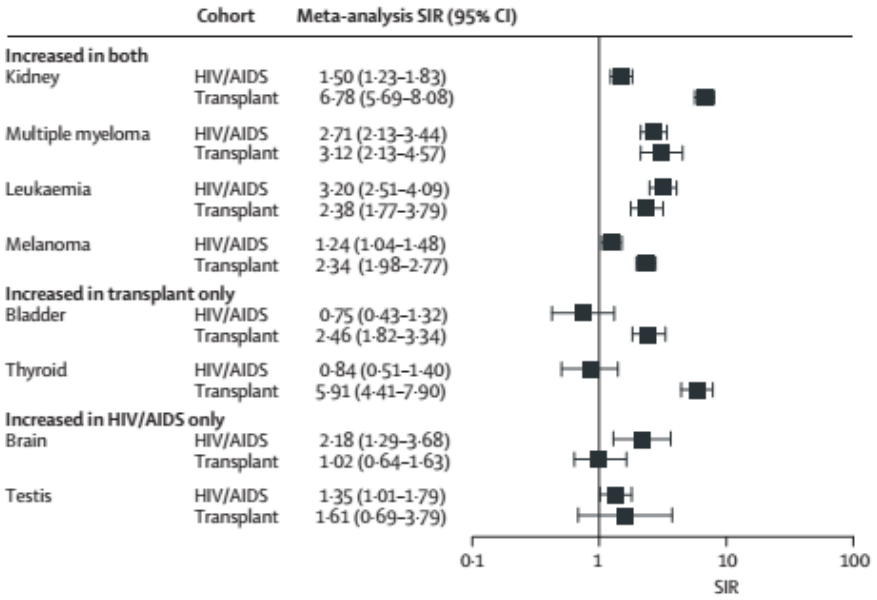

Figure 1. Standardized incidence ratios for non-AIDS cancers increased in one or both immunosuppressed cohort populations (Adapted from Grulich et al.[15])

1.2 The Polyomaviridae family

a) Identification history

Back in 1953, the first virus in this family was discovered, named murine polyomavirus and able to induce multiple (*poly*-) tumors (*-oma*) in new-born mice[17]. In 1960, simian virus 40 (SV40) was isolated from rhesus monkeys kidney cells used to produce polio vaccines[18]. Due to the carcinogenic ability of SV40 in animals, and its worldwide inoculation to humans through SV40-contaminated polio vaccines, it became a public health concern[19]. First human polyomaviruses were BK (BKPyV) and JC (JCPyV) polyomaviruses in 1971, isolated in urine and brain tissue, respectively[20,21]. 36 years later, by implementation of improved molecular biology techniques, new polyomaviruses were and continue to be discovered at an increasing rate. In 2007, KI (KIPyV) and WU (WUPyV) polyomaviruses were identified in respiratory

samples of symptomatic children[22,23]. In 2008, MCPyV in 2008, was identified from targeted Merkel cell carcinoma cells[24]. In 2010, 3 more viruses were identified; HPyV6 and HPyV7 were found in skin samples[25] while Trichodysplasia spinulosa-associated polyomavirus (TSPyV) was isolated from an infected hair follicle of a subject with this hairy disorder[26]. In 2011, HPyV9 was finally isolated in a serum sample from a renal transplant subject[27] and in a skin swab sample[28], a long time suspected to exist virus due to an up to 30% human seroresponse against antigens from the lymphotropic polyomavirus (LPyV), whose natural host is the African green monkey. In 2012, 3 research groups identified the same virus, the Malawi polyomavirus (MWPyV), in stool samples from Malawi[29] and Mexico[30] and in skin samples of a United States subject with a rare genetic disease[31]. Later in 2012, St Louis polyomavirus (STLPyV) was identified in stool samples from the United States and Gambia[32]. Early 2013, the twelfth human polyomavirus, HPyV12, was identified in liver samples[33].

b) Viral structure, genome organization, life cycle and phylogeny

Polyomaviruses are non-enveloped small (40-50 nanometers diameter) viruses with a circular double stranded DNA of around 5000 base pairs (see Figure 2) and three functional regions.

The non-coding region (NCCR), contains the origin of replication and a bidirectional promoter-enhancer region containing several transcription binding sites. The early region encodes the large tumor antigen (LT-Ag) and the small tumor antigen (sT-Ag) proteins, which would regulate the viral DNA replication, and will be the first to be transcripted. Subsequently, in a regular life cycle, through a not yet elucidated mechanism, the replication direction is switched into the late region transcription. This region encodes the three VP structural proteins, that will auto assemble together encapsidating the viral genome inside. Viral particles will then be released for new cell infection by cell lysis.

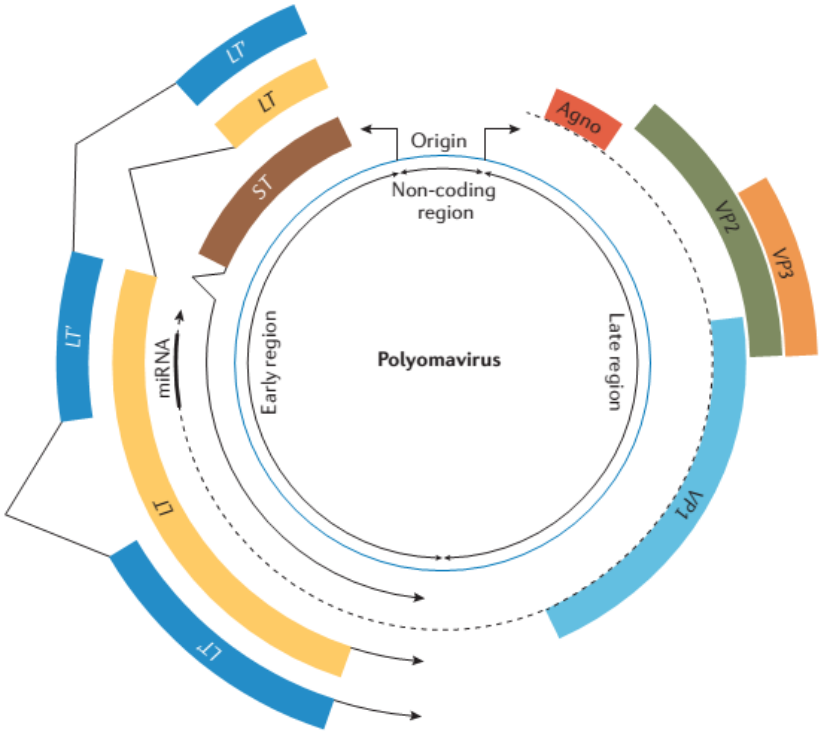

Figure 2. Human polyomaviruses genomic organization (Adapted from DeCaprio and Garcea[34])

All human polyomaviruses have the same three regions and encode for the same proteins, although their length, even between strains from the same polyomavirus specie, can vary depending on alternative splicing of the mRNA transcript. Additionally, BKPyV and JCPyV also encode for a small protein called agnoprotein, within the late region, whose function remains elusive[35]. Also, these two polyomaviruses and MCPyV[36] encode their own miRNA, which autoregulate the early genes expression and have also been involved in immune evasion mechanisms[37,38].

The phylogenetic classification of polyomaviruses (see Figure 3) within the *Polyomaviridae* family is modelled according to degree of similarity of genetic sequence comparison. Viruses at close positions within the tree are likely to share highly conserved regions

and, therefore share functionalities such as host cell tropism and carcinogenicity potential.

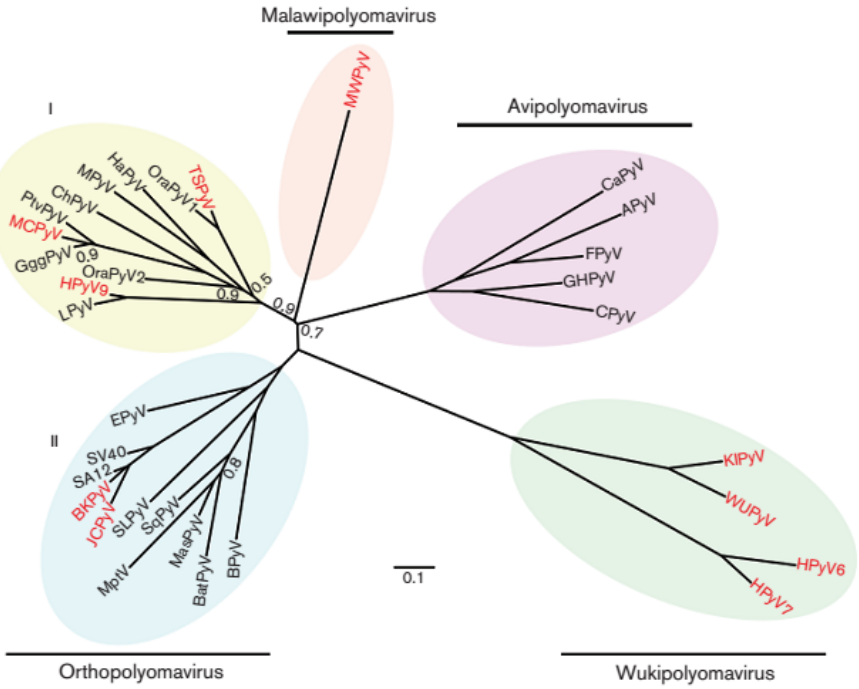

Figure 3. *Polyomaviridae* family phylogenetic tree
Tentatively suggested by Feltkamp et al.[39], in agreement although enlarging the number of genera described by Johne et al. in 2010[40]. Letters in red indicate those with human tropism.

c) Natural history of polyomaviruses

Data on polyomaviruses seroprevalences, as indicator of human-tropic infection instead of a possible environmental contamination, is available for all polyomaviruses except for STLPyV. However, a divergent variant of STLPyV has been isolated from skin warts, which suggests a potential ability to infect skin cells[41] and therefore, human tropism.

Overall, high seroprevalences (>50%) are already observed at young ages for all polyomaviruses except for HPyV7, HPyV9 and HPyV12[33,42–45]. Some variability is observed between studies, probably related to the population characteristics and/or

measurement technology used further discussed in section 4.4 of the Methodology section[34]. In adult population (aged 20 or older), seroprevalences up to 95% can be reached for some viruses although showing different behaviors between them. Increasing seroprevalences at increasing age are observed for JCPyV, WUPyV, HPyV6, HPyV7 and MCPyV[42–46]. BKPyV shows an increasing seroprevalence trend until around 50, when it starts to decrease[43–46]. KIPyV[43] and HPyV12[33] show quite stable results, although while KIPyV has an overall 60% seroprevalence, HPyV12 is around 25% seroprevalence. HPyV9 overall remains stable at lower values[45,47] although Nicol et al. detected an increase with age in Italian population[42]. Regarding TSPyV, whereas stable seroprevalence was observed in The Netherlands[48], others have observed an slightly increasing trend[45,46]. Recent data in MWPyV indicates a peak at age 3-4 with 70% seroprevalence that decreases until 30 and then remains stable at around 45%[42].

Since no symptoms or diseases have been linked to any polyomavirus infection during childhood, it is generally accepted that polyomavirus cause an asymptomatic primary infection followed by either a latent (no replication at all) or a persistent (very low replication levels) asymptomatic infection until its potential reactivation.

Overall, the transmission routes and latency sites remain unknown, although based on the biological and environmental samples where polyomaviruses DNA have been detected, some suggestions have been done. A summary of samples and tissues tested is included in Table 2. First column includes samples where the viruses were isolated or those where viruses prevalence is higher. Second column includes other samples where the viruses have been detected at lower rates.

BKPyV and JCPyV are quite well characterized regarding latency/persistence site of infection and cell tropism. The data in the first column for these viruses include tissues where cell receptors that allow viral entry are expressed. Furthermore, based on intermittent excretion of BKPyV and JCPyV in urine, a persistent rather than a latent infection in reno-urinary tract has been suggested.

Table 2. Summary of polyomaviruses detection in samples and tissues

Virus	*Latency / persistence infection site*	*Other samples with positive detection*
BKPyV	Epithelium cells of kidney, ureter, bladder and urethra (reno-urinary tract)	Tonsils, skin, brain, bone, colon (and feces), prostate and female genital tract, sperm and white blood cells
JCPyV	B-lymphocytes in tonsils and spleen, oligodendrocytes and astrocytes, and kidney and lung tissue	
KIPyV **WUPyV**	Respiratory tract samples and also lung tissue for KIPyV	Tonsils, lymphoid tissue, feces, blood and also cerebral spinal fluid for WUPyV
MCPyV	Skin	Respiratory tract, saliva, lymphoid tissue, urine and gastrointestinal tract
HPyV6 **HPyV7**	Skin?	Nasopharyngeal swabs, feces (HPyV6) and urine (HPyV7)
TSPyV	Trichodysplasia spinulosa lesion	Skin, eyebrow hair, nasopharyngeal swabs, feces, kidney biopsy and urine
HPyV9	Blood?	Urine, throat swabs and skin
MWPyV **STLPyV** **HPyV12**	Feces?	Nasal washes and skin (MWPyV); Urine and skin warts (STLPyV)

Created from reviews in [49–55] and Pastrana et al.[41]

On the other hand, the lack of expression of JCPyV viral proteins in normal brain tissue[56] suggests a non-replicative latent infection in brain[57]. Regarding MCPyV, both viral DNA and encapsidated virions are detected and chronically shed in skin samples[25,58] suggesting it as primary infection site. As per the other polyomaviruses, very little is known; few different samples have been analyzed and further studies would be necessary.

Therefore, potential respiratory or oro-fecal transmissions are suggested for BKPyV, JCPyV, KIPyV, WUPyV and MCPyV. As well, skin contact transmission for MCPyV, HPyV6 and HPyV7 is suggested. As per the other polyomaviruses, information is too scarce to hypothesize potential transmission routes.

Regarding environmental samples, Bofill-Mas et al. analyzed sewage and river samples searching for polyomaviruses DNA. The samples were obtained near Barcelona, where the epidemiologic studies used in this thesis have recruited subjects. They obtained positive results for JCPyV, BKPyV[59] and MCPyV[60], suggesting a potential urine and/or fecal excretion and therefore potential transmissions by ingestion of contaminated water or of uncooked or undercooked food. On the other hand, Foulongne et al.[61] looked for MCPyV DNA in several environmental surface samples in contact with human skin. Positive detection was observed in 85% of the samples tested, a third of these remaining positive after DNase treatment, suggesting a potential transmission by contact to these surfaces.

d) Polyomaviruses and non-cancer diseases

To date, clinical manifestations have been observed mainly in adult population under immunosuppression conditions, and therefore believed to be caused by viral reactivation. Furthermore, the viral immunosuppression linked to the HIV pandemia, the immunosenescence due to increased life expectancy and the increasing use of drug-induced immunosuppression to treat cancer, autoimmune diseases and to avoid transplant rejection, are the suspected causes of the substantially increase in the number of cases linked to polyomaviruses reported in the last half century[62].

Briefly[63], non-cancer pathologies caused by polyomaviruses are a consequence of the cytopathic loss of infected cells by a highly active viral replication, with (i.e. BKV-associated hemorrhagic cystitis) or without (i.e. Progressive multifocal leukoencephalopathy) an inflammatory response related to necrosis and/or by a dominant inflammatory response against an abundance of viral antigen, typically following a brisk recovery of the cellular immune response (i.e, Polyomavirus associated nephropathy).

To date, three viruses have been associated with clinical diseases:

- *JCPyV and progressive multifocal leukoencephalopathy (PML)*

PML was the first disease to be linked to a polyomavirus, JCPyV, in 1971[21].

Neither viral pathogenesis nor activation mechanisms have been elucidated. However, rearranged NCCR (rr-NCCR) sequences have been observed predominantly in brain, plasma and bone marrow samples from PML subjects while the archetype NCCR is mainly observed in the reno-urinary tract[64]. These variants with the rr-NCCR have an increased early gene expression and higher replication rate[65]. It has been suggested that these characteristics can be related to neurotropism and to a poorer clinical outcome.

Three potential pathways of viral reactivation and subsequent pathogenesis have been suggested by Hirsch et al.[64]; (i) the initial infection takes place in the central nervous system (CNS) awaiting for a potential reactivation, (ii) the initial infection takes place in susceptible cells outside the CNS, is reactivated and the virus then migrates to the CNS or (iii) the initial infection takes place in plasma cells, that migrate and remain latent in the CNS until reactivation. Furthermore, since these rr-NCCR are thought to emerge from the archetype NCCR, Bellizzi et al.[66] have hypothesized a potential rearrangement taking place in B-cell lymphocytes, since these cells are susceptible of infection and contain enzymes required for immunoglobulin rearrangements.

Besides the previously mentioned immunosuppressing settings that could lead to viral reactivation, PML has also been observed in subjects with hematological malignancies although it is most

frequently diagnosed in HIV infected subjects[67]. In a cohort of HIV positive subjects, after implementation of antiretroviral therapy, the incidence was estimated in 0.06 cases (95%CI: 0.04–0.10) per 100 person-years.

First described in 1958[68], PML is a progressive neurological deficit consistent with a hemispheric or posterior fossa localization. After a subacute onset, frequent clinical symptoms include hemiparesis, ataxia, visual disturbances or higher cortical dysfunction, such as dysphasia or agnosia[69,70]. Diagnosis, as stated recently by the American Association of Neurology Neuroinfectious Disease Section, requires of both histopathological confirmation by imaging and JCPyV detection[71].

- *BKPyV and polyomavirus-associated nephropathy (PyVAN)*

Although isolated in 1971[20], the association with PyVAN was not confirmed until 1999[72,73].

As observed for JCPyV, rearranged NCCR variants have been detected in urine, plasma and biopsies of patients with BKPyV diseases. *In vitro* studies suggest the archetype strain as the transmissible form, while the rearranged one would be associated with disease progression[74]. Furthermore, although BKPyV replicates poorly in *in vitro* cultures, it has been observed an efficient replication in a human embryonic cell line that contains a high expression of SV40 LT-Ag[75] suggesting a potential role of other co-infections in BKPyV replication[76]. Additionally, since the disease is rarely observed in organ transplant recipients other than kidney, where it is observed in 1-10% subjects after 2 years post-transplant, specific factors linked to renal transplant are suspected to be involved in BKPyV reactivation.

First described by Purighalla in 1995[77], PyVAN is the consequence of the cytopathic loss of renal-tubular epithelial cells in the transplanted kidney, which can allow the virus to leak into tissue and bloodstream. Therefore, inflammatory cells can infiltrate the interstitium leading to tubular atrophy and interstitial fibrosis[76] Diagnosis requires tissue confirmation of cytopathic damage and BKPyV presence confirmation by immunohistochemistry or *in situ* hybridization.

The worsening of disease stage is linked to a potential increasing graft loss from <10% in pattern A subjects to >80% in pattern C[78]. Therefore, the following screening scheme is recommended by the Kidney Disease: Improving Global Outcomes (KDIGO) Transplant Work Group; BKPyV quantitative PCR in plasma monthly the first 3-6 months and then every three months until the end of the first year after transplantation. Also, in case of unexplained rise in serum creatinine and after treatment for acute rejection[79].

- *BKPyV and BKPyV-induced hemorrhagic cystitis*

The association between the virus and the disease is dated in the mid 80s[80–82].

As for BKyVAN, its appearance is linked to transplant recipients, although it mainly occurs in allogenic hematopoietic stem cell transplant recipients (5-15%)[76]. Pathogenesis has not been elucidated, although Leung et al.[83] suggested that during the post-engraftment, the return or development of immunity against BKPyV would cause extensive mucosal damage and hemorrhage.

The disease symptoms can include dysuria, frequency and urgency of urination, suprapubic pain, bladder spasms and varying degrees of hematuria. Mild cases would resolve within 2 weeks of supportive care while severe ones can develop severe pain, uncontrollable bleeding, acute renal failure and prolonged hospitalization[84]. No guidelines or recommendations exist regarding diagnosis. However, it would require of post-engraftment occurrence, cystitis, hematuria (at least grade II) and high BKPyV urine loads. Other infections, such as cytomegalovirus, as well as other bleeding disorders should be ruled out. Apparently, monitoring of plasma BKPyV could be useful since it has been correlated to clinical recovery[76,85].

- *TSPyV and trichodysplasia spinulosa (TS)*

In 1999, Haycox et al. observed intracellular viral particles in pathological skin biopsies by electron microscopy[86] consistent with those from the *Papovaviridae* family. However, it was not until 2010 when the viral genome of these particles could be isolated, sequenced and therefore, TSPyV identified[26].

Since the association is quite recent, very little is known on the disease pathogenesis. However, four times higher TSPyV DNA loads have been observed in trichodysplasia lesions when compared to controls[87].

TS is a rare disease, with only around 30 cases reported to date, in solid organ transplant subjects or subjects with hematological malignancies[55].

First described in 1995[88], is also known as trichodysplasia, pilomatrix dysplasia of immune suppression, cyclosporine-induced folliculodystrophy, or viral-associated trichodysplasia[55]. It is characterized by a gradual development of papules and spicules (spines) on the face, sometimes accompanied by eyebrows and lashes alopecia. Histopathology findings show abnormal follicles with excessive inner root sheath differentiation[89]..

1.3 Polyomavirus and cancer

a) Experimental studies

Most of the experimental (*in vitro* and animal) data on the oncogenic ability of polyomaviruses are based on SV40. Its LT-Ag is able to inactivate the retinoblastoma protein (pRB) and p53 tumor suppressor family members[90]. On the other hand, SV40 sT-Ag is able to interact with the protein phosphatase 2A (PP2A) family[91]. SV40 LT-Ag is able to induce cell transformation by itself but co-expression of SV40 sT-Ag, which alone is not able to induce transformation, enhances LT-Ag transformation ability[92].

Recently, MCPyV has become the focus of attention for experimental studies, showing differences with the known SV40-transformation model. MCPyV sT-Ag has transformation ability, can induce transformation by itself and is not apparently enhanced by co-expression of MCPyV LT-Ag. Furthermore, MCPyV sT-Ag interaction with PP2A is not likely to play a primary role in oncogenesis. MCPyV LT-Ag expression alone is not sufficient for cell transformation, but its targeting of pRB is necessary for survival and growth of tumor cells. Additionally, MCPyV contains an LT sequence truncation that prevents interaction with p53[93–95].

Although viruses are host-specific and this fact restricts the potential of tumor development in non-natural hosts, the inoculation of SV40, BKPyV and JCPyV in animals has been observed to generate a variety of tumors. In animals, JCPyV causes mainly brain tumors[51] whereas BKPyV has been associated with several types, such as brain, kidney, pancreas and bone and soft tissue tumors[49]. SV40 has been able to develop brain and bone tumors, mesothelioma and lymphomas[96].

b) Merkel cell carcinoma

First described in 1972[97], it is a rare aggressive skin cancer whose incidence, in the United States, has been increasing in the last decades[98]. Merkel cell carcinoma characteristics are summarized in the *AEIOU* acronym; *A*symptomatic disease with a rapid *E*xpansion in subjects *I*mmunosuppressed and/or *O*lder than 50 years old, associated with *U*ltraviolet exposure[99]. In Denmark, survival rates at 1 year since diagnosis were estimated of 78% for subjects with localized disease and 46% for non-localized disease, decreasing to 45% and 16% at 5 years, respectively[100]. Most cases are cytokeratin 20 (CK20) positive although rare negative variants have been reported[101].

MCPyV is not ubiquitously present in all Merkel cell carcinoma cases. When MCPyV-negative versus –positive cases are compared, different gene expression profiles[102] and microRNA patterns[103] have been observed. Better outcomes have been observed in MCPyV-positive Merkel cell carcinoma cases[104,105], although not observed by others[106]

Viral DNA is integrated into the host cell genome[24] at no preferential site[107], and although Wetzels et al.[108] have reported the presence of viral particles within cells, replication and virion formation is unlikely to occur. All MCPyV sequences isolated from Merkel cell carcinoma samples contain a deletion of the origin-binding or helicase domain of the LT-Ag that prevents viral replication[93]. Additionally, mutations in the noncoding origin sequence[109], that also prevents replication, or in the VP1 structural gene[110], that prevents capsid formation, have been reported. Therefore, two mutations have been suggested to cause

the tumor development; (i) an initial one that will cause the viral genome integration into the host genome and (ii) a second one that would inhibit viral replication[111].

- *Epidemiological and virological evidence of MCPyV in Merkel cell carcinoma*

MCPyV is detected in around 80% of Merkel cell carcinoma cases, as originally observed by Feng et al.[24]. In a review of published data[112], overall presence of MCPyV, estimated using different DNA and/or antigens detection techniques, was of 74% in a total of 2354 pooled Merkel cell carcinoma samples. Individual studies prevalence of MCPyV DNA ranged between 24% in Autralia[113] and 100% detection in USA[114], Korea[115], Italy[116], France[117] and Finland[118]. Regarding LT-Ag expression, rates range between 18% in Australia[119] and 97% in Canada[120]. In Merkel cell carcinoma samples, positive for MCPyV LT-Ag, no expression of VP1 protein was detected[121].

Of note, Rodig et al.[122], using an extended repertoire of PCR primers, detected viral DNA in 100% of CK20-positive Merkel cell carcinoma cases, 8% of them with less than 0.1 copies per cell. Furthermore, using a self-developed monoclonal antibody with an increased sensitivity, named Ab3, 97% of the samples showed positive results for LT-Ag, including the samples with low DNA copy number. The same samples tested using CM2B4, the monoclonal antibody used in previously described prevalence studies, detected an 81% LT-Ag expression suggesting a potential 100% attributable fraction to MCPyV. Although no negative control samples were used, lung cancer samples tested negative. However, Xie et al.[103], using Ab3 monoclonal antibody, detected an 81% LT expression in 26 CK20-unknown Merkel cell carcinoma samples from Sweden.

Serology measurements against VP1 structural protein are not exclusive of Merkel cell carcinoma cases, although higher seroprevalences[123–125] and higher seroreactivities[44,121,125,126] have been observed in cases when compared to healthy subjects. In contrast, serology against LT-Ag and sT-Ag is almost specific for Merkel cell carcinoma cases, with 30% and 40% seroprevalences

respectively, in comparison to around 1% seroprevalence for both antigens in healthy subjects[123].

In a recent prospective study, Faust et al.[127] have shown an increased risk of subsequent Merkel cell carcinoma diagnosis among subjects with MCPyV neutralizing antibodies and high antibody titers in samples collected 12 years (range: 1-26 years) before diagnosis.

c) Epidemiologic studies in humans

- *SV40*

There are a large number of papers published regarding SV40 detection in cancer, mainly involving mesotheliomas, brain tumors, osteosarcoma and NHL, since these tumors develop in animal models after SV40 inoculation. However, most cohort and case-control studies published have not found any increased incidence or prevalence of cancer among polio vaccinated population with SV40-contaminated batches neither among those SV40 seroprevalent[128].

Regarding previously published case-series[96,129], their interpretation is controversial because of the conflicting data and limited reproducibility between research groups for the same tumor type. In a study involving nine laboratories, the International SV40 Working Group assessed and obtained a high sensitivity, specificity and reproducibility of SV40 DNA detection methodology. However, consistent negative SV40 presence in mesothelioma samples and positive detection in negative controls raised concerns regarding potential contamination in previous studies that obtained SV40 positive results in mesothelioma samples[130]. This was supported by López-Ríos et al.[131], who tested mesothelioma samples with four different sets of primers, used in previous studies, and demonstrated that these primers provided false positive results due to contamination with SV40 plasmids.

Therefore, although carcinogenicity data for SV40 in animals is strong, since there is no convincing evidence of carcinogenic effect in humans, the IARC classified it as group 3 (not classifiable as to its carcinogenicity to humans)[2].

- *BKPyV and JCPyV*

Most of published epidemiological studies have assessed these two polyomaviruses together and mainly in the same tumors studied for SV40. Again, no clear association has been observed[128] except for JCPyV and colorectal cancer, whose data is controversial[132].

Nested case-control studies showed no differences in BKPyV or JCPyV seroprevalences and subsequent development of colorectal cancer in men[133,134]. However, among these studies, Rollison et al.[134] observed a decreased risk in women to develop colorectal cancer and adenomatous polyps but a higher risk of adenoma in men. Furthermore, when Lundstig et al.[133] used an alternative and supposedly more accurate cutpoint, a significant decreased risk of colorectal cancer in JCPyV seroprevalent men was obtained.

As observed for SV40, there is a large number of case-series that looked for DNA or LT-Ag of BKPyV and JCPyV[49,51], Prevalence estimates are largely variable between studies. Part of these differences could be linked to geographic differences or due to methodological issues, such as the primers used for DNA detection.

The IARC evaluation of the carcinogenicity of BKPyV and JCPyV classified both viruses as group 2B (possibly carcinogenic to humans) based on inconsistent evidence for association with various humans cancers and sufficient evidence in animals[2].

- *MCPyV*

Besides Merkel cell carcinoma, most studies in MCPyV have focused in CLL and other tumors sharing common features with Merkel cell carcinoma, such as other skin or neuroendocrine cancers. Most promising results have been obtained for CLL (further discussed in section 1.4.b) and squamous cell carcinoma (SCC).

Although a varying DNA prevalence (0-38%) has been observed in SCC subjects[135,136], and DNA loads have been quantified as more than 100x lower than those observed in Merkel cell carcinoma cases[137], a higher seroreactivity has been observed in SCC cases compared to healthy controls using a case-control study[136]. Dworkin et al.[138], additionally, detected a MCPyV DNA nucleotide mutation

likely to result in a truncated LT-Ag, such as that observed in Merkel cell carcinoma cases, in all tumor samples from 14 SCC subjects

Several case-series have analyzed different types of cancer but generally there is only one or two studies per cancer type which do not provide evidence enough to judge potential causality. However, most of the studies have shown low DNA prevalence suggesting an unlikely association.

Based on the limited evidence observed in humans, inadequate evidence in experimental animals and strong mechanistic evidence in humans, MCPyV was classified by the IARC as group 2A (probably carcinogenic to human)[2].

- *Other polyomaviruses*

Very few studies have analyzed these viruses, mainly included as co-infections in previously described studies. Among those few published, null or low presence of viral DNA has been detected.

1.4 Potential cancer targets

Based on the potential carcinogenicity of polyomavirus and their natural history, this thesis focuses on the evaluation of the association between human polyomaviruses in cancer. More specifically, (i) MCPyV with 11 specific subtypes of lymphoproliferative malignant neoplasms, (ii) BKPyV, JCPyV and MCPyV with bladder cancer and (iii) nine human polyomaviruses (BKPyV, JCPyV, LPyV, KIPyV, WUPyV, HPyV6, HPyV7, TSPyV and MCPyV) with chronic lymphocytic leukemia,.

Descriptive epidemiology and related previous studies are detailed specifically for each neoplasm.

a) Lymphoproliferative disorders

The term lymphoproliferative disorders refers to several conditions with an excessive production of lymphocytes, including the lymphoid neoplasms or lymphomas. The latter are clonal tumors of mature and immature B cells, T cells and natural killer (NK) cells at various stages of differentiation, individually defined according to

morphologic, immunophenotypic, genetic, molecular and clinical features[139].

Lymphomas are commonly stratified according to their cell lineage (B-cell vs. T-cell/NK) and maturation stage. Among B-cell lineage, more frequent entities are chronic lymphocytic leukemia (CLL), diffuse large B-cell (DLBCL) and follicular (FL) lymphomas. Although Hodgkin lymphoma (HL) derives from B-cell lymphocytes it has been treated as a separate category, and therefore lymphomas have been traditionally stratified in HL and NHL (non-Hodgkin lymphoma). Additionally, previous studies in NHL may have studied both B and T cell lymphomas together whereas multiple myeloma (MM) may or may not have been included within the NHL category.

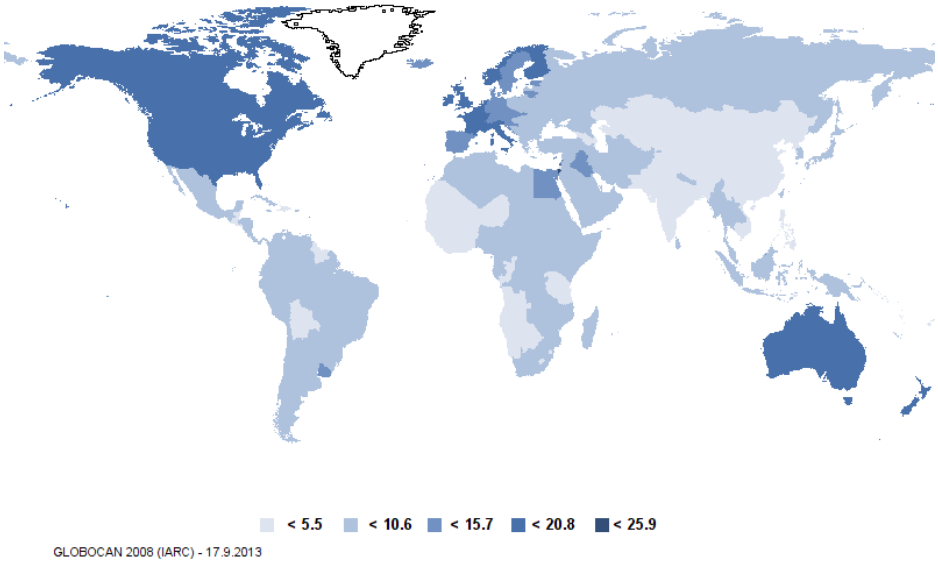

Figure 4. Estimated age-standardized incidence rates per 100.000 inhabitants of lymphoma (all HL, NHL and MM) for both sexes

These malignancies are more frequently registered in more developed world regions than in less developed ones. According to data from GLOBOCAN[140], estimated age-standardized incidence rates (by grouping data for HL, NHL and MM, which do not include CLL) in 2012 reached up to 24.1 new cases in Israel per 100.000 inhabitants (see Figure 4).

Main risk factors for overall NHL include infections (see Table 1), personal history of autoimmune disease and family history of hematological disease. Other potential risk factors with inconsistent results include pesticides, use of hair dyes, high BMI and smoking[141,142]. However, the known risk factors listed above do no explain the increased incidence of NHL in the last decades. Additionally, NHL includes diverse heterogeneous lymphoma subtypes that difficult the interpretation of the results when studied together[143]. Regarding HL, main risk factors include EBV and genetic susceptibility[144].

Although some infections have already been linked to lymphomas, they do not fully explain the 11x and 77x higher incidence of HL and NHL, respectively, observed in HIV/AIDS population[15]. The detection of polyomaviruses in lymphocytes and lymphoid tissue, as well as the lymphomagenesis caused by SV40 in animals, suggest polyomaviruses as potential infectious candidates in lymphoma development.

- *Previous studies on polyomaviruses and lymphoproliferative disorders*

Epidemiological studies in SV40, BKPyV and JCPyV, did not observe, overall, any association with NHL. In cohort studies.[145,146], no differences in NHL incidence were observed between exposed and unexposed to SV40 contaminated vaccines. In nested case-control studies, previous SV40, BKPyV or JCPyV seroprevalence were not associated with NHL[147,148]. Using case-control studies, no increased risk of NHL was observed for previous reported history of polio vaccination[149] neither for SV40 seroprevalence[150–152], which substantially decreased after pre-incubation with BKPyV and/or JCPyV in competitive serology assays indicating a strong cross-reactivity between these three viruses[153]. However, although no differences were observed for BKPyV seroprevalence, a decreased risk of NHL was observed among JCPyV seroprevalent subjects[154].

In stratified analyses of BKPyV and JCPyV by lymphoma subtype, Rollison et al.[148], in a nested case-control study, did not observe any association between viral seroprevalence and subsequent risk of DLBCL or FL. Engels et al.[154], in a case-control study, did not

observe differences for BKPyV seroprevalence, but a decreased risk of FL, DLBCL and T-cell lymphomas among JCPyV seropositive subjects although only significant for DLBCL (OR=0.66; 95%CI:0.48-0.91).

Regarding MCPyV, no epidemiological studies have been published in lymphomas but a few case-series. Shuda et al.[155], using commercial and non-commercial tumor tissue samples, obtained low DNA prevalences (<5%) for DLBCL, FL and HL. DNA copy number were 2-4x lower than those observed in Merkel cell carcinoma cases, and among 144 additional lymphoma samples none expressed MCPyV LT-Ag. Andres et al.[156], in 23 samples from 19 subjects with cutaneous lymphoma, identified only 4 positive samples to at least one of two MCPyV strains. However, among two samples from the same subject, only one of the samples was positive for both strains. Toracchio et al.[157] obtained an overall 6.6% MCPyV DNA presence in lymphomas (B-cell lymphoma: 6%, T/NK-cell lymphoma: 11% and Hodgkin lymphoma: 6.8%). Among 11 lymphomas (unknown subtype), only one angioimmunoblastic T-cell lymphoma sample tested positive for MCPyV LT-Ag expression. Teman et al.[158] did not detected MCPyV DNA in 17 FL cases.

b) Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is an heterogeneous disease, considered as both lymphoma and leukemia, characterized by a clonal expansion of B-lymphocytes in blood, bone marrow, lymph nodes and/or spleen[159]. Its age-standardized rate of over 30 cases per 100 000 inhabitants[12] in more developed countries, defines it as the most frequent leukemia and a 7% of all lymphomas. It is more frequently observed in males than in women (ratio 2:1) and in subjects aged over 65 years old.

Etiology of CLL is unknown but some risk factors have been identified. An increased risk of CLL is observed among subjects with family history of hematologic disease, especially among siblings, those that used hair dyes before 1980, pesticides and genetic susceptibility factors[160–163]. Other potential risk factors include occupational exposures, such as workers exposed to

nonionizing electromagnetic field and farmers. Contradictory data has been shown for smoking and history of rheumatoid arthritis[164].

Besides the reasons observed for a potential association of polyomaviruses with lymphomas, Merkel cell carcinoma occurs at a higher incidence than expected in subjects with a prior diagnosis of CLL and vice versa[100,165,166]. Therefore, this direct association between CLL and Merkel cell carcinoma suggests a shared etiology by MCPyV.

- *Previous studies on polyomaviruses and CLL*

BKPyV and JCPyV studies in lymphoproliferative disorders[148,154], did not stratify to analyze a potential specific effect in CLL subjects. MCPyV has been further explored, and overall, the case-series analyzed do not support a potential association. Up to 35% of MCPyV DNA or LT-Ag has been detected in tissue or PBMC at lower viral DNA loads than those observed in Merkel cell carcinoma subjects[155,157,158,167–169]. Nevertheless, although Pantulu et al.[170] detected MCPyV DNA only in 19 (25%) out of 70 CLL samples, six of these positive samples showed a truncated LT-Ag sequence, subsequently confirmed to be located in the nucleus[171], as observed for Merkel cell carcinoma. MCPyV seroprevalence has been only tested previously by Tolstov et al.[172] who obtained a 55% MCPyV seroprevalence in 18 CLL subjects.

HPyV9 is the only polyomavirus of those newly identified, different than MCPyV, that has been analyzed in CLL, but among 25 b-cell CLL subjects none of them was positive for viral DNA[169].

c) Bladder cancer

Bladder cancer is referred to malignancies arising from the epithelium of the urinary bladder, or urothelium. Based on the cell of origin, two main histopathologic types can be identified; urothelial cell carcinoma (UCC), formerly known as transitional cell carcinoma, and squamous cell carcinoma (SCC), the latter mainly caused by *S. haematobium*.

Based on updated data from 2012, Spain has the 7th highest estimated age-standardized rate worldwide, with 13,9 cases (26,0 in males and 3.7 in females) per 100.000 inhabitants[173] (see Figure 5).

Regarding the histopathologic type, different distributions are observed worldwide; UCC is observed in around 90% of the subjects in industrialized countries, whereas up to 50% of the subjects are diagnosed with SCC in east Africa and Middle East countries, in agreement with *S. haematobium* infection prevalence[174].

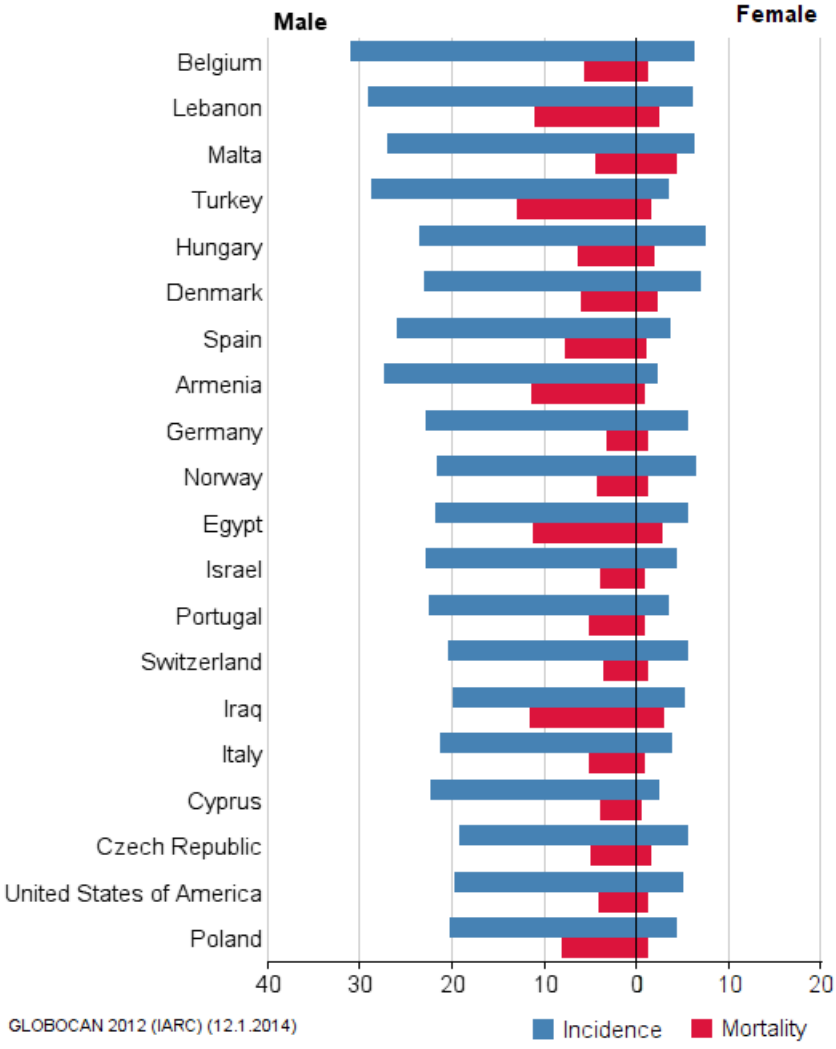

Figure 5. Estimated age-standardized incidence and mortality rates per 100.000 inhabitants of bladder cancer, by sex.

Tobacco consumption is the main risk factor of UCC. Aromatic amines and other chemicals occupational exposure, certain analgesics, water contaminants ingestion and genetic susceptibility factors are also important contributors to bladder cancer burden. No infectious etiology has been identified yet for UCC, the most frequently observed in Spain, but an increased risk of cancer has been observed in reported cases of cystitis and other urinary tract infections[175,176].

As observed in Figure 1, an increased incidence of bladder cancer has been observed in organ-transplant population, enhancing a potential infectious etiology. Since polyomaviruses can cause urinary tract infections or be detected in urine and reno-urinary tract samples, they are likely candidates to fill this etiologic gap.

- *Previous studies on polyomaviruses and bladder cancer*

Previous studies mainly include several case-series, case reports and two epidemiological studies. In Italy, two studies detected BKPyV DNA in around 55% of the 26 and 32 bladder cancer samples tested[177,178] whereas in the US, the largest case-series to date with 74 bladder cancer subjects only detected 5% of positive cases. On the other hand, Newton et al.[179] in a nested case-control did not observe an association of BKPyV seroprevalence and subsequent risk of bladder cancer, but data was only based on 9 cases. Furthermore, Polesel et al.[180] did not observe any association for BKPyV, JCPyV, MCPyV, KIPyV or WUPyV DNA with bladder cancer in a case-control study but viral detection was performed in urine samples and viruria is considered as a biomarker of active replication. Besides the study from Polesel et al.[180], MCPyV has only been tested by Loyo et al.[181], who detected 6 out of 8 MCPyV DNA positive bladder cancer samples but none in 2 bladder normal tissue samples.

2. RATIONALE

- Several infections have been observed to play a major role in carcinogenesis through different mechanisms. Among them, associations between EBV and HCV in lymphomas and *S. haematobium* in bladder cancer have been identified but these do not fully explain the increased burden in immunosuppressed subjects (see section 1.1).

- Little is known about polyomaviruses. They are common infections (up to 95% of the population infected), isolated from a large variety of human samples and tissues, including lymphoid tissue and urinary tract. Its associated diseases occur mainly under an immunosuppressing setting, leading to the conclusion of viral reactivation of childhood acquired latent/persistent infections (see section 1.2).

- Among them, Merkel cell polyomavirus has been associated with Merkel cell carcinoma. BK and JC polyomavirus are known to produce cancer in animals. The remaining polyomaviruses, although not fully studied, are considered as potentially carcinogenic (see section 1.3).

- Potential associations studies between polyomaviruses and human cancer, other than Merkel cell carcinoma, have increased in the last years but epidemiological data is contradictory and inconclusive (see section 1.3), including that for lymphomas and bladder cancer (see section 1.4).

3. OBJECTIVES

The main objective of the present thesis is to assess the potential causal association of different polyomaviruses with cancer in Spain.

Specific objectives to pursue the main objective have been:

- ✓ To assess the association between MCPyV seroprevalence and seroreactivity with eleven lymphoma subtypes within the Epilymph case-control study.

- ✓ To assess the association between BKPyV, JCPyV and MCPyV seroprevalence and seroreactivity with bladder cancer within the Epicuro case-control study.

- ✓ To assess the association between nine polyomaviruses (BKPyV, JCPyV, LPyV, KIPyV, WUPyV, HPyV6, HPyV7, TSPyV and MCPyV) seroprevalence and seroreactivity with chronic lymphocytic leukemia within the Multicase control Spain study.

4. METHODOLOGY

This section contains an overview of the methodology used, further detailed in each manuscript.

4.1 Study population

Three case-control studies have been used in this thesis; the Epilymph study on lymphomas, the Epicuro study in bladder cancer and the Multicase control Spain study (MCC-Spain study) with the collaboration of the International Cancer Genome Consortium (ICGC) in CLL. Details on the study population included are summarized in Table 3.

Table 3. Details of the subjects included in the studies

Study	*Cases*	*Control population*
Epilymph (1998 – 2002)	Incident lymphoma	* Hospital-based * Frequency match by age, sex, and recruitment center
Epicuro (1998 – 2001)	Incident untreated carcinoma of the urinary bladder	* Hospital-based * Individual matched by age, sex, ethnic origin and recruitment center
MCC-Spain + ICGC (2008 - …)	* Incident breast, colorectal, gastric and prostate cancer * Incident and prevalent CLL	* Population-based * Frequency match by age, sex, and recruitment center * Common set of controls for all pathologies

A personal interview by trained personnel to collect questionnaire data was performed to the participating subjects of each study. Epidemiologic data collected included socio-demographics, lifestyle habits, previous medical and medication history, family history of cancer, occupational and residential histories and other environmental exposures. Blood samples were also collected.

Additional data collected in the MCC-Spain study includes a self-completed diet questionnaire, anthropometric measures as well as saliva, hair and nail samples.

Among these studies, all subjects available with blood sample were selected to participate in our analyses. On June 2012, the MCC-Spain study was still recruiting CLL cases and therefore all available cases with blood sample, at that moment, and a potential control match were selected. Regions participating in the three studies are detailed in Figure 6.

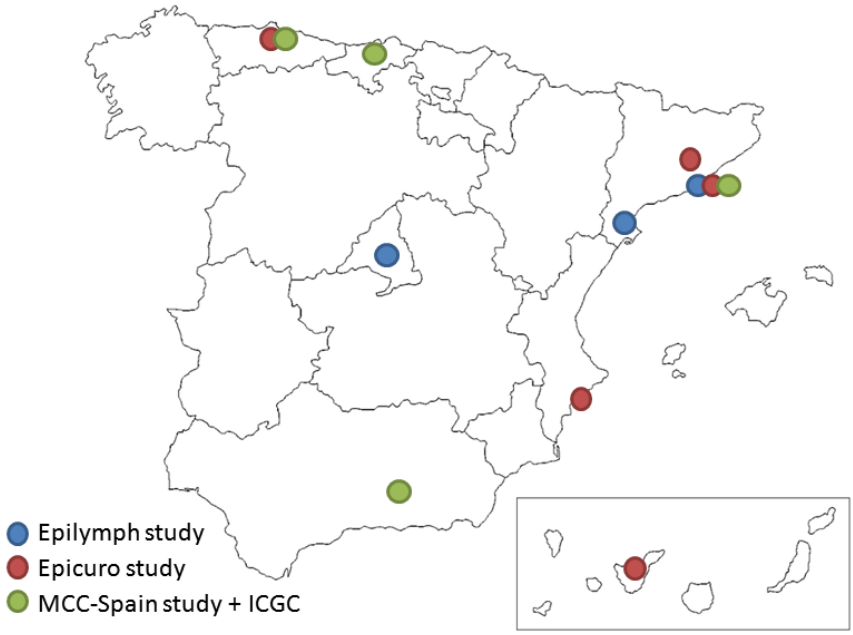

Figure 6. Regions participating in the analyses

Additionally, on paper I, a sub-study was done on DLBCL cases and two randomly selected controls matched by sex, age and center.

4.2 Exposure measurement

Viral exposure was measured by detection and quantification of human seroreactivity (antibodies) against viral antigens in serum samples using two different techniques.

On one hand, enzyme immunoassays (EIA or ELISA) measuring seroreactivity against viral virus-like-particles (VLP) was used. Analyses were performed at the John Hopkins School of Public Health (JHSPH; Baltimore, USA) and exposure was provided as optical density. This technique was used in papers I (MCPyV) and II (BKPyV, JCPyV and MCPyV).

Additionally, in paper I sub-study, seroreactivity against BKPyV and JCPyV VLP were measured in selected samples. Also, MCPyV antibody levels using endpoint titration were obtained as EIA units.

On the other hand, for paper III, fluorescent bead-based multiplex serology measuring seroreactivity against GST-antigens was used. Analyses were performed in the German Cancer Research Center (DKFZ; Heidelberg, Germany) and exposure was provided as median fluorescent intensity. Specifically, seroreactivity against VP1 capsid protein was measured for BKPyV, JCPyV, LPyV, KIPyV, WUPyV, HPyV-6, HPyV-7, TSPyV and MCPyV. Seroreactivity against oncoproteins LT-Ag was measured for JCPyV, TSPyV and MCPyV, whereas it was only measured against sT-Ag for MCPyV.

For paper I and II, cut-off values (COV) defining seroprevalence were established mathematically as an OD greater than the mean seroreactivity results plus 4 SDs of unexposed serum samples. For paper III, COVs were identified by visual inspection of the data obtained in previous studies done by the laboratory.

For analyses purposes, continuous data on seroreactivity was categorized into tertiles based on seroprevalent control subjects.

4.3 Statistical analyses

Potential confounding factors were identified by assessing differences in study population and viral seroprevalence using chi2 test, or Fisher exact test when applicable. Correlation between continuous data, such as viral seroreactivity and age, were assessed by Spearman coefficient.

Associations between viral seroprevalence and target cancer were assessed by logistic regression. In matched data, unconditional logistic regression was used, unless otherwise specified that conditional logistic regression was used. In polytomous outcomes, such as lymphoma subtype or disease stage, multinomial logistic regression was preferred. All analyses were adjusted at least by matching variables.

Cancer associations with seroresponse were performed using seroreactivity categorized into tertiles based on control data distribution using unconditional logistic regression. Trend effect was assessed by use of the categorical variable as continuous. Generalized additive models (GAM) were used to evaluate the exposure–response curve.

Potential interactions were evaluated by stratification and introduction of cross-product terms in the logistic regression models.

Significance level was established at 0.05 and all tests were two-sided. Analyses were conducted with Stata software, version 10.1.

4.4 Viral exposure validation

Discrepancies in published age-specific seroprevalence data against the viral capsid (i.e. VP1 in polyomaviruses) have been generally justified by the methodology used to detect the virus or the population under analysis.

In the present thesis, two different methodologies have been used to measure viral exposure; VLP EIA and GST-antigen fluorescent bead-based multiplex serology. Main differences are the antigen analyzed and the quantity of antigens tested at the same moment.

Regarding the antigen, VP1 proteins have been traditionally expressed using recombinant baculoviruses in insect cells that self-assemble into a pseudo viral capsid called virus-like-particles (VLP). However, in the recent years, a recombinant VP1 protein conjugated with glutathione-S-transferase (GST) has been expressed in *Escherichia coli*. This methodology increases the

production of VP1 protein, its solubility and further facilitates the protein purification[182]. However, GST-VP1 auto-assemble into pentamers (small aggregates of 5 VP1 proteins) instead of VLP.

Both proteins can be measured by EIA and data obtained for both proteins has been previously compared. Bodaghi et al.[183] observed a higher detection and seroreactivity of BKPyV VLP in comparison to BKPyV GST-VP1, justifying these differences due to the loss of the three-dimensional conformation in GST-VP1. However, data is based on 12 samples and no statistic test is provided. On the other hand, although based on HPV-16, Sehr et al.[184] obtained a kappa test value of 0.62 and a linear regression coefficient of determination of R^2=0.68 while Davidson et al.[185] obtained a significant linear correlation (r=0·85, P<0·001) for GST-L1 and L1 VLP.

As per the number of antigens tested, EIA fixes the antigen to a plate so that only one antibody-antigen reaction can take place. To increase specificity and get a refined measurement, competitive pre-incubation analysis with other antigens can be done. On the other hand, the multiplex assay fixes the antigen to beads that can be added into an up to 100 beads suspension mixture, each one containing a different antigen. Therefore, it allows for multiple testing at the same time, resembling a competitive pre-incubation assay, in less time and at a lower sample quantity expense.

Previous studies have provided similar results between bead-based fluorescence serology and EIA. As an example, Waterboer et al.[186] measured HPV16 GST-L1 using both methods and obtained a kappa value of 0.85 and a correlation coefficient of 0.94.

A subgroup of Epilymph samples, originally selected to study another infectious association, were analyzed by both methodologies in the JHSPH and the DKFZ. Tested samples include subjects with DLBCL, CLL and their matched controls. Data on concordance and correlation of measurements were estimated.

In the JHSPH, as detailed in section 4.2, MCPyV was measured in all samples whereas BKPyV and JCPyV only in the sub-study to further study DLBCL and selected matched controls. In the DKFZ,

the same testing than that used in the MCC-Spain study was performed and therefore nine polyomaviruses were measured. The number of tested samples by each and both laboratories is provided at Table 4.

Table 4. Number of samples tested by polyomaviruses

	JHSPH	DKFZ	BOTH
MCPyV			
Controls	552	202	192
DLBCL	83	87	81
CLL	108	115	107
BKPyV and JCPyV			
Controls	157	202	82
DLBCL	82	87	80

Concordance between categorized data according to each method was estimated using the kappa index. Because of the lack of normal distribution, Spearman's coefficient (rho) was estimated to study the correlation between individual measurements.

Furthermore, the association of polyomaviruses (BKPyV, JCPyV and MCPyV) with DLBCL and CLL using DKFZ data to replicate the results in study I was performed. Data is provided as an addendum to section 5.1.

In control population, kappa coefficients of concordance regarding seroprevalence categorization between methodologies were of 0.57 for BKPyV, 0.62 for JCPyV and 0.62 for MCPyV. Kappa values in DLBCL and CLL subjects were similar except for BKPyV among DLBCL cases, decreasing to a kappa value of 0.29. According to magnitude guidelines[187], agreement between categorized seroprevalences can be graded as moderate / good in control population.

High correlation of continuous data was observed. Spearman's rho was estimated around 0.85 for BKPyV and JCPyV in control and DLBCL population. A slightly lower correlation (rho= ~0.74) for MCPyV in CLL, DLBCL and control population was observed.

Therefore, data measured by both methodologies provide similar results. A good correlation was obtained for all viruses and type of subject studied. The lower kappa value observed for BKPyV in DLBCL is likely explained by differences in the selected COV instead of differences in assay sensitivity.

4.5 Tasks performed at this thesis

Recruitment on the Epilymph and Epicuro studies was finalized several years ago. Databases were already clean and almost ready to be analyzed. Therefore, main tasks related to this thesis included bibliographic search, data analysis and manuscript writing.

However, participation in the MCC-Spain study has also included general participation in different steps of the study. Tasks have involved obtaining occasionally signed informed consent and biological samples from study participants, review of patient's file to obtain clinical data, help in questionnaire interview and data cleaning.

Specifically for the purposes of this thesis regarding the MCC-Spain study analysis, selection of cases and participation in control selection was done. Logistics issues to get the blood samples analyzed for viral exposure were solved, including contract agreement, contact with sites, centralization of samples in Barcelona and shipment to Germany. Initial purpose was to participate in the measurement of the samples during the 3-month stay at the DKFZ but serological analyses were delayed. However, participation in other samples analyses, similar to the present ones, was done to understand the full process of viral exposure measurement.

4.6 Funding

This thesis would not have been possible without the grants listed below. None of the funding sources played a role in the study design, data collection, analysis or interpretation and reporting of provided results.

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- Others:
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5. RESULTS

The main findings described in this thesis are:

PAPER 1 - Antibody Response to Merkel Cell Polyomavirus Associated with Incident Lymphoma in the Epilymph Case–Control Study in Spain

- ✓ MCPyV seroprevalence was associated with a 6 times higher risk of diffuse large b-cell lymphoma (DLBCL). BKPyV and JCPyV were not associated.

- ✓ MCPyV median seroreactivity, measured by means of endpoint titration instead of optical density units, was almost significantly higher in DLBCL subjects when compared to control subjects.

- ✓ Other lymphomas, including CLL, showed overall higher MCPyV seroprevalences than controls, although non-significant.

- ✓ Categorized viral seroreactivity was only significantly associated with multiple myeloma by means of a reverse causality effect due to the inability of these subjects to produce a proper immune response.

PAPER 2 - Bladder cancer and seroreactivity to BK, JC and Merkel cell polyomaviruses: The Spanish bladder cancer study

- ✓ None of the three polyomaviruses tested (BKPyV, JCPyV and MCPyV) showed an association between viral seroprevalence and bladder cancer risk.

- ✓ An increasing risk of bladder cancer was observed at increasing BKPyV and MCPyV seroreactivity, but not for JCPyV seroreactivity.

- ✓ No differences in bladder cancer phenotype were observed for any of the associated viruses, suggesting an unlikely relation between polyomavirus seroreactivity and disease severity.

- ✓ Viral associations were not modified by smoking status

PAPER 3 – Seroreactivity against Merkel cell polyomavirus and other polyomaviruses in chronic lymphocytic leukemia, the MCC-Spain study

- ✓ All nine polyomaviruses analyzed showed lower VP1 seroprevalences in CLL subjects, suggesting a reverse causality effect due to disease-related immunosuppression.

- ✓ In both controls and CLL subjects, LT-Ag seroprevalences (MCPyV and TSPyV) and sT-Ag (MCPyV) seroprevalence were almost null.

- ✓ No differences in seroprevalence were observed by disease severity, but significant lower seroprevalences could already be observed at low stages of disease severity.

- ✓ Among subjects with stages I-IV, treated subjects showed higher JCPyV VP1 and LT-Ag seroprevalences, suggesting a potential viral reactivation.

5.1 Paper I

Robles C, Poloczek A, Casabonne D, Gonzalez-Barca E, Bosch R, Benavente Y, Viscidi RP and De Sanjose S.

Antibody Response to Merkel Cell Polyomavirus Associated with Incident Lymphoma in the Epilymph Case–Control Study in Spain

Cancer Epidemiol Biomarkers Prev 2012;21(9):1592–8.
DOI: 10.1158/1055-9965.EPI-11-1140

Addendum

Further analyses were performed on DLBCL subjects. These results will not be published but are provided here as part of the thesis since they provide relevant insight to the associations observed.

- *Replication of MCPyV results in DLBCL and CLL subjects*

As detailed in section 4.4, some samples from the Epilymph study were tested both by EIA and fluorescent bead-based multiplex serology. Statistical analyses using the multiplex serology data were done as in Paper I. All control population was compared to each lymphoma subtype using multinomial logistic regression adjusting by match variables.

Study population description did not show any relevant differences in comparison to those observed in Paper I (see Supplemental Table I). Again, differences in familiar history of cancer and blood transfusion for CLL were observed. No differences in gender were observed in CLL because of the matched-paired case-control design, but the older population in CLL subjects raised differences in age distribution for DLBCL. A significant lower number of DLBCL subjects had ever lived in a rural area in comparison to controls. MCPyV seroprevalence descriptive in control population did not identify any risk factor of viral infection. Full descriptive can be found in Table 1 within this addendum.

Association analyses of MCPyV seroprevalence with DLBCL and CLL provided similar results to paper I. Slightly lower risks were obtained for CLL (OR=1.60; 95%CI=0.86-3.00) and for DLBCL (OR=4.73; 95%CI=1.87-11.96) among MCPyV seroprevalent subjects but remained significant for DLBCL (see Table 2 within this addendum).

Median seroreactivity among seroprevalent subjects showed no differences for DLBCL (median=4892.6, p=0.21) and CLL (median=5007.6, p=0.28) when compared to control population (median=4251.1). Categorized data into tertiles based on data distribution in control population did not show any significant trend.in cancer risk at increasing seroreactivity.

Table 1. Detailed characteristics of study population and of MCPyV seroprevalence among control population

	Control		DLBCL		CLL		Control		
								MCPyV sero(+)	
	n	%	n	%	n	%	Total	n	%
Overall	202	(100)	87	(100)	115	(100)	202	157	(77.7)
Sex									
Men	116	(57.4)	42	(48.3)	74	(64.3)	116	91	(78.4)
Women	86	(42.6)	45	(51.7)	41	(35.7)	86	66	(76.7)
p-value			*p=0.152*		*p=0.227*				*p=0.774*
Center of recruitment									
Barcelona	148	(73.3)	57	(65.5)	91	(79.1)	148	116	(78.4)
Madrid	36	(17.8)	20	(23.0)	16	(13.9)	36	26	(72.2)
Tarragona	18	(8.9)	10	(11.5)	8	(7.0)	18	15	(83.3)
p-value			*p=0.412*		*p=0.507*				*p=0.634*
Age									
17-56	51	(25.2)	38	(43.7)	12	(10.4)	51	36	(70.6)
57-68	55	(27.2)	16	(18.4)	41	(35.7)	55	45	(81.8)
69-74	51	(25.2)	14	(16.1)	36	(31.3)	51	41	(80.4)
75-87	45	(22.3)	19	(21.8)	26	(22.6)	45	35	(77.8)
p-value			***p=0.012***		***p=0.013***				*p=0.524*
p-trend									*p=0.436*
Level of studies									
Primary or none	162	(80.2)	62	(71.3)	96	(83.5)	162	127	(78.4)
Secondary	20	(9.9)	12	(13.8)	10	(8.7)	20	17	(85.0)
University	20	(9.9)	13	(14.9)	9	(7.8)	20	13	(65.0)
p-value			*p=0.245*		*p=0.758*				*p=0.299*
p-trend									*p=0.338*
Type of cancer antecedent									
None	137	(67.8)	51	(58.6)	51	(44.3)	137	104	(75.9)
Hematologic	4	(2.0)	3	(3.4)	8	(7.0)	4	2	(50.0)
Non Hematologic	61	(30.2)	33	(37.9)	56	(48.7)	61	51	(83.6)
p-value			*p=0.294*		***p=0.000***				*p=0.145*
Siblings*									
No	9	(4.5)	5	(5.7)	2	(1.8)	9	8	(88.9)
Yes	193	(95.5)	82	(94.3)	112	(98.2)	193	149	(77.2)
p-value			*p=0.639*		*p=0.208*				*p=0.365*
Previous blood transfusion									
No	142	(71.7)	63	(73.3)	99	(87.6)	142	41	(73.2)
Yes	56	(28.3)	23	(26.7)	14	(12.4)	56	114	(80.3)
p-value			*p=0.790*		***p=0.001***				*p=0.277*
Ever lived in rural area									
No	69	(34.2)	46	(52.9)	50	(43.5)	69	54	(78.3)
Yes	133	(65.8)	41	(47.1)	65	(56.5)	133	103	(77.4)
p-value			***p=0.003***		*p=0.099*				*p=0.895*

Table 2. Association between MCPyV with DLBCL and CLL.

		Positive				
	Total	**n**	**(%)**	**OR**	**95%CI**	**p-value**
MCPyV seroprevalence						
Control	202	157	(77.7)	1	ref.	
DLBCL	87	81	(93.1)	**4.73**	**(1.87-11.96)**	***0.001***
CLL	115	98	(85.2)	1.60	(0.86-3.00)	*0.140*

- *Testing for DNA, RNA and cDNA in DLBCL samples (by others)*

Because of the strong association observed between MCPyV and DLBCL, we looked for original biopsies from the Epilymph study obtained during initial study recruitment.

5 frozen DLBCL samples could be recovered and sent to the Centro Superior de Investigación en Salud Pública in Valencia for genetic material extraction. DNA and RNA were extracted. cDNA was obtained by retro-translation of RNA previously treated with DNase.

Extracted material was sent to JHSPH for MCPyV detection. Using the primers described by Bhatia et al.[188]. All samples were negative, but these results are based on a small sample size. Furthermore, although the methodology used is quite sensitive, others[122] have described a more sensitive one.

These results are likely reflecting a null presence of MCPyV in DLBCL samples or viral presence at a low copy number under one copy per cell.

5.2 Paper II

Robles C, Viscidi R, Malats N, Silverman DT, Tardon A, Garcia-Closas R, Serra C, Carrato A, Herranz J, Lloreta J, Rothman N, Real FX, De Sanjose S and Kogevinas M.

Bladder cancer and seroreactivity to BK, JC and Merkel cell polyomaviruses: the Spanish bladder cancer study.

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5.3 Paper III

Robles C, Casabonne D, Benavente Y, Costas L, Gonzalez-Barca E, Aymerich M, Campo E, Tardon A, Jiménez-Moleón JJ, Castaño-Vinyals G, Dierssen-Sotos T, Michel A, Kranz L, Aragonés N, Pollan M, Kogevinas M, Pawlita M and De Sanjose S

Seroreactivity against Merkel cell polyomavirus and other polyomaviruses in chronic lymphocytic leukemia, the MCC-Spain study

Submitted

SHORT COMMUNICATION

Title of manuscript:
Seroreactivity against Merkel cell polyomavirus and other polyomaviruses in chronic lymphocytic leukemia, the MCC-Spain study

Authors and affiliations:
Claudia Robles[1,2], Delphine Casabonne[1,3], Yolanda Benavente[1,3], Laura Costas[1,3], Eva Gonzalez-Barca[4], Marta Aymerich[5], Elias Campo[5], Adonina Tardon[3,6], José J. Jiménez-Moleón[3,7,8], Gemma Castaño-Vinyals[2,3,9,10], Trinidad Dierssen-Sotos[3,11], Angelika Michel[12], Lena Kranz[13], Nuria Aragonés[3,14,15], Marina Pollan[3,14,15], Manolis Kogevinas[2,3,9,10,16], Michael Pawlita[13] and Silvia de Sanjose[1,3]

1. Unit of Infections and Cancer (UNIC), Cancer Epidemiology Research Programme, Institut Catala d' Oncologia – Catalan Institute of Oncology, IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain.
2. Universitat Pompeu Fabra (UPF), Barcelona, Spain
3. CIBER Epidemiología y Salud Pública (CIBERESP), Spain.
4. Hematology Department, Institut Catala d' Oncologia – Catalan Institute of Oncology, IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain.
5. Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Universitat de Barcelona, Barcelona, Spain.
6. Instituto Universitario de Oncología, University of Oviedo, Spain.
7. Department of Preventive Medicine and Public Health, University of Granada
8. Instituto de Investigación Biosanitaria de Granada, Servicio Andaluz de Salud/Universidad de Granada, Spain.
9. Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain
10. IMIM (Hospital del Mar Research Institute), Barcelona, Spain.
11. Facultad de Medicina, Universidad de Cantabria – IDIVAL, Santander, Spain

12. Infections and Cancer Epidemiology, Infection and Cancer Program, German Cancer Research Center (DKFZ), Heidelberg, Germany
13. Department of Genome Modifications and Carcinogenesis, Infection and Cancer Program, German Cancer Research Center (DKFZ), Heidelberg, Germany.
14. Environmental and Cancer Epidemiology Unit, National Center of Epidemiology, Instituto de Salud Carlos III, Madrid, Spain.
15. IIS Puerta de Hierro, Majadahonda, Spain.
16. National School of Public Health, Athens AL, Greece.

Running title: Case-control study of Polyomaviruses and CLL

Key words: polyomavirus, case-control, seroepidemiology, chronic lymphocytic leukemia, Spain

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ABSTRACT

BACKGROUND AND OBJECTIVE
Chronic Lymphocytic Leukemia (CLL) etiology is largely unknown. Evidence on a potential role of previously acquired common infections is inconsistent. We evaluated the role of 9 polyomaviruses in CLL etiology using serological data.

METHODS
We recruited 359 CLL cases and 370 randomly selected population controls frequency-matched by sex, age and recruitment area in four regions of Spain. CLL cases were classified into 204 Rai 0 and 145 Rai I-IV. Seroreactivities against BKPyV, JCPyV, LPyV, KIPyV, WUPyV, HPyV-6, HPyV-7, TSPyV and MCPyV VP1 capsid and T antigens were measured using bead-based multiplex serology technology. Odds ratio (OR) and 95% confidence intervals (95%CI) for CLL and disease stages associated with seroprevalence and seroreactivity were estimated using logistic regression models.

RESULTS
High seroprevalences (69-99%) in the control population for all human polyomaviruses were observed, which contrasted with low viral seroprevalences among cases. Cases showed a non-significant lower MCPyV seroprevalence (OR=0.79, 95%CI=0.54-1.16) compared to controls. Significant inverse associations with CLL were observed for other polyomavirus seroprevalences evaluated (OR range=0.21-0.70). Results did not change by disease stage.

CONCLUSION
CLL cases showed a reduced ability to amount seroresponse against polyomaviruses. This phenomenon, already observed at low Rai stages, suggests a potential early impairment of the immune response.

MANUSCRIPT

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is one of the most common B-cell malignancies in Europe with an incidence rate around 5 cases per 100,000 (1). It shows a 2:1 male/female ratio and a higher frequency in the elderly, with a median age of 65-72 years at CLL diagnosis (2). An increased risk of CLL is consistently observed among those with family history of hematological malignancies. Other suspected risk factors include long term use of pesticides and regular use of hair dyes (2–4). However, its etiology remains unknown and infections have been suggested as potential candidates.

Recently, Merkel cell polyomavirus (MCPyV) has been identified as an oncogenic virus responsible of Merkel cell carcinoma, a rare skin tumor. Interestingly, Merkel cell carcinoma incidence has been reported to be higher among CLL patients and vice versa (5), suggesting a shared etiology. However, whereas low prevalences (2-36%) of viral DNA and large-T antigen (LT-Ag) were detected in tumor tissue or peripheral blood mononuclear cells of CLL subjects (6–11), nuclear presence of truncated LT-Ag sequence, a peculiarity of MCPyV in MCC samples, was detected in highly purified CD19+/CD5+ CLL cells (12,13). Additionally, in a previous case-control study we observed a non-significant increased risk of CLL in MCPyV seropositives (14).

To date, besides MCPyV, 11 additional human polyomavirus have been identified (BKPyV, JCPyV, KIPyV, WUPyV, HPyV6, HPyV7, trichodysplasia spinulosa-associated polyomavirus -TSPyV-, HPyV9, Malawi polyomavirus –MWPyV-, St Louis polyomavirus –STLPyV- and HPyV12) (15,16). BKPyV and JCPyV have shown in vitro carcinogenic effects and can infect lymphocytes, which could lead to a potential transformation into CLL. The remaining polyomaviruses preserve the molecular characteristics responsible of cell transformation and therefore remain potentially carcinogenic.

In this study, we aimed to explore the seroreactivity of MCPyV and 8 additional polyomaviruses in CLL patients and controls within the context of a large multicentre case-control study in Spain.

METHODS

CLL cases were recruited within the MCC-Spain study (www.mccspain.org), an epidemiological population-based multicase-control study, in collaboration with the International Cancer Genome Consortium on Chronic Lymphocytic Leukemia Project (ICGC-CLL; www.cllgenome.es), previously described elsewhere (17–19).

For the present study, a case-control study frequency-matched by sex, region and age at interview (+/-5 years) by random selection was performed. Eligible subjects included, incident and prevalent CLL cases and population based controls with no prior history of lymphoproliferative disorder recruited between 2010 up to July 2012 from 7 centers within Asturias, Barcelona, Cantabria and Granada regions. The final study population included 370 controls and 359 CLL cases (by disease stage: 204 Rai 0, 145 Rai I-IV and 10 unclassifiable). Ethical approval and subject informed consents were obtained.

Infectious exposure measurement

Serostatus of antibodies against 12 viral proteins from 8 human polyomaviruses were determined; BKPyV (capsid antigen - VP1), JCPyV (VP1 & LT-Ag), KIPyV (VP1), WUPyV (VP1), HPyV-6 (VP1), HPyV-7 (VP1), TSPyV (VP1 & LT-Ag) and MCPyV (VP1, LT-Ag & small T antigen – sT-Ag). Additionally, antibodies to African Green Monkey lymphotropic polyomavirus (LPyV) VP1, closely related and highly cross-reactive with HPyV-9 were determined. Measurement was performed using multiplex serology, a glutathione S-transferase capture immunosorbent assay combined with fluorescent-bead technology, as described elsewhere (20,21). Bead sorts, each carrying a different antigen, were mixed and incubated with human sera at 1:1000 dilutions. Antibodies bound to the beads via the viral antigens were stained by biotinylated anti-human-IgG and streptavidin-R-phycoerythrin. Beads were examined in a Luminex 100 analyzer that identifies the bead color of each bead sort and quantifies the antibody bound to viral antigen via the median R-phycoerythrin fluorescence intensity (MFI) of at least 100 beads of the same internal color. Seroprevalence cut-off values were based on previously arbitrarily defined cut-off values

(22) by visual inspection of cumulative histograms of antibody reactivities (MFI) as described before (20) and applied implying a quality control panel of assay serum standards run in both studies. Cut-offs were set to 250 MFI for VP1 proteins, 400 MFI for large T antigens and 200 MFI for small T antigen of MCPyV except for BKPyV VP1 which was adjusted to 100 MFI because of decreased reactivity in comparison to other polyomavirus VP1 seroreactivities.

Statistical analysis

Odds ratio (OR) and 95% confidence interval (95%CI) were used to estimate the association with antigen seroprevalence by means of unconditional logistic regression for all cases together and multinomial logistic regression for stratified disease, both adjusted by sex, region and age (quartiles according to control distribution). Treated cases (n=73) were considered as a separate entity. Case-case analyses were performed to assess differences between disease stages among untreated subjects. Sensitivity analyses were done by exclusion of prevalent cases (n=131) diagnosed more than 3 years prior to study interview. Significance level was established at 0.05 and all tests were two-sided. Analyses were conducted with Stata software, version 10.1.

RESULTS

Study population description is detailed in Table 1. CLL cases showed a 1.6:1 male/female ratio and mean age of 67 years (range=41-88). CLL cases were associated with a familiar history of hematological neoplasms (p=0.04). By disease stage, CLL Rai I-IV cases were less likely to be current smokers (p=0.04). No other associations were observed for viral seroprevalence in controls (Supplemental Table 1), and therefore analyses were only adjusted for frequency-matched variables.

The associations between CLL and polyomavirus VP1 seroprevalences are shown in Table 2. Overall, seroprevalences in control population ranged between 70 and 99%, except for LPyV (44%). Cases consistently showed lower seroprevalences, translated into statistically significant lower OR of CLL (OR range=0.21-0.79) for all polyomaviruses except for MCPyV (p-value=0.23). By disease stage, the results were very similar both in terms of magnitude and direction.

Regarding seroprevalence against other polyomaviruses antigens, no CLL cases were positive for TSPyV LT-Ag or MCPyV LT-Ag. MCPyV sT-Ag seroprevalence was observed only in one CLL case and one control subject. Therefore, analyses were only performed for JCPyV LT-Ag including 45 seropositive subjects (Table 2). As observed for VP1, inverse associations between JCPyV LT-Ag seropositivity and CLL, overall and by disease stages, were observed.

Raw data stratified by disease stages were summarized using box-plots (Figure 1). CLL cases, irrespective of disease stage, overall showed lower VP1 seroreactivity levels, leading to lower VP1 seroprevalences than controls. When seroreactivity levels were explored (Supplemental Table 2), CLL risks decreased with increasing seroreactivity irrespective of diseases stage. The analysis showed similar results when prevalent subjects were excluded.

DISCUSSION

Our data identified that VP1 seroreactivity to different polyomaviruses was considerably reduced among CLL patients as compared to population controls. The inverse association observed for CLL Rai 0 suggests an impaired immune response already present at low stages of disease. Additionally, no CLL cases showed MCPyV LT-Ag seroprevalence and only one case showed MCPyV sT-Ag seroprevalence.

Results likely reflect a reverse causality effect in which CLL cases are impaired to amount a proper immunological response. However, it is unknown whether it reflects a quantitative (low production) or a qualitative (inadequate binding antigen-antibody) disorder. The lower seroprevalences, already observed at CLL Rai 0 cases, suggest that in mild asymptomatic stages a CLL-related immunodeficiency is likely to be present. The lack of strong associations between age and polyomaviruses seroprevalences in the control population discards a potential effect of age-related immunosuppression. Antonsson et al. (23), did not observe any trend in JCPyV and BKPyV seroreactivity measured after 11 years of follow-up.

Regarding MCPyV, in a previous study we observed a non-significant increased risk between CLL and MCPyV seroprevalence

(OR=1.49; 95%CI=0.80-2.77) (14). This discrepancy could be attributable to differences in study design, such as the previous use of hospital-based controls and a higher presence of younger controls. If MCPyV plays a role in CLL, as observed for Merkel cell carcinoma, an increased VP1 seroprevalence, increased VP1 seroreactivity and an almost exclusive LT-Ag and sT-Ag seroprevalence would be expected in CLL cases (24). Therefore, our lower MCPyV VP1 seroreactivity, the almost null seroprevalence of MCPyV LT-Ag and MCPyV sT-Ag, and the previously published data in CLL cases (6–11), suggest that MCPyV is unlikely to play a role in CLL development. Regarding previous discrepant studies encouraging an association between MCPyV and CLL, no further studies have been published on the mutated MCPyV LT-Ag sequence detected in highly purified CLL cells (12,13). On the other hand, the increased incidence of CLL in patients with Merkel cell carcinoma and vice versa (5), could be explained by a necessary and shared immunosuppressive status to enhance both diseases development although at different progression rates.

Interestingly, higher seroprevalences were observed for JCPyV VP1 and LT-Ag among treated CLL Rai I-IV cases versus controls suggesting that, even under a CLL-related immunosuppression setting, treatment could lead to potential viral reactivation. Specifically for JCPyV, this could imply a subsequent development of progressive multifocal leukoencephalopathy, an opportunistic disease with an increased incidence in CLL subjects (25).

This is the largest CLL study looking for a potential association with polyomaviruses, using a population based case-control design. The use of serologic biomarkers reduces the potential misclassification of exposure, a frequent limitation in retrospective studies. However, a prospective design would be preferable to assess the immune response against infectious agents before the CLL-related immunosuppression starts.

We conclude that the lower seroreactivities to MCPyV and other polyomaviruses tested in CLL patients potentially reflect an underlying immunosuppression. Further studies on polyomaviruses infection may be relevant to better understand the role of CLL-immunosuppression in the immunosurveillance of latent infections.

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CONFLICT OF INTEREST
The authors declare no conflict of interest

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Table 1. Descriptive characteristics of study population

	Control		All cases*		Stratified			
					Rai 0		Rai I-IV	
	n	%	n	%	n	%	n	%
Sex								
Men	230	(62.2)	221	(61.6)	120	(58.8)	95	(65.5)
Women	140	(37.8)	138	(38.4)	84	(41.2)	50	(34.5)
				p=0.867		*p=0.475*		*p=0.543*
Center of recruitment								
Barcelona	285	(77.0)	280	(78.0)	176	(86.3)	97	(66.9)
Asturias	48	(13.0)	45	(12.5)	23	(11.3)	20	(13.8)
Cantabria	19	(5.1)	16	(4.5)	3	(1.5)	12	(8.3)
Granada	18	(4.9)	18	(5.0)	2	(1.0)	16	(11.0)
				p=0.972		***p=0.005***		***p=0.029***
Age								
40-61	90	(24.3)	84	(23.4)	43	(21.1)	40	(27.6)
62-67	83	(22.4)	83	(23.1)	44	(21.6)	35	(24.1)
68-74	95	(25.7)	90	(25.1)	57	(27.9)	32	(22.1)
75-79	102	(27.6)	102	(28.4)	60	(29.4)	38	(26.2)
				p=0.981		*p=0.792*		*p=0.755*
Level of studies								
Less than primary	114	(30.8)	108	(32.0)	67	(36.0)	39	(27.5)
Primary	100	(27.0)	100	(29.7)	59	(31.7)	35	(24.6)
Secondary	98	(26.5)	79	(23.4)	35	(18.8)	44	(31.0)
University	58	(15.7)	50	(14.8)	25	(13.4)	24	(16.9)
				p=0.739		*p=0.140*		*p=0.697*
Previous cancer								
None	327	(88.9)	289	(85.5)	159	(85.5)	122	(85.9)
Yes	41	(11.1)	49	(14.5)	27	(14.5)	20	(14.1)
				p=0.182		*p=0.274*		*p=0.364*
Family history of cancer								
None	83	(25.1)	97	(30.3)	59	(33.7)	36	(26.5)
Non-hematotological cancer	221	(66.8)	184	(57.5)	91	(52.0)	87	(64.0)
Hematological cancer	27	(8.2)	39	(12.2)	25	(14.3)	13	(9.6)
				p=0.039		***p=0.003***		*p=0.782*
Smoking status								
Never Smoker	173	(47.0)	159	(47.3)	91	(49.2)	65	(45.8)
Current smoker	59	(16.0)	39	(11.6)	25	(13.5)	12	(8.5)
Ex-smoker	136	(37.0)	138	(41.1)	69	(37.3)	65	(45.8)
				p=0.198		*p=0.748*		***p=0.041***

Heterogeneity test performed by means of chi-square test for all cases and Fisher exact test in recategorised cases, versus controls.

** Include 10 cases that could not be further stratified*

Table 2. Association between polyomavirus seroprevalence and CLL; all cases and stratified by disease stage

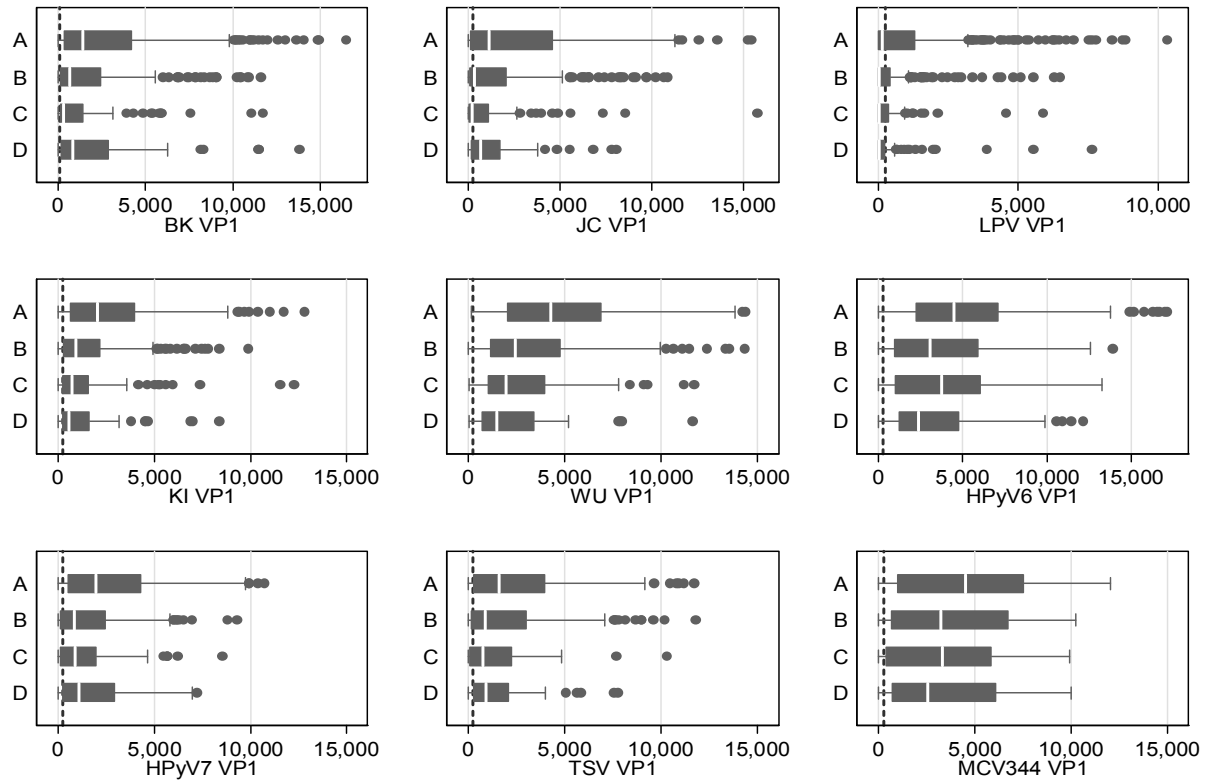
	Controls		All cases[a] (n=359)					Stratification by rai stage[b]									Treated[d] (n=73)			
								CLL Rai 0 (n=204)				CLL Rai I-IV (n=72)								
	N	%	N	%	OR	95% CI	*p-value*	N	%	OR	95% CI	N	%	OR	95% CI	*p(het)* [c]	N	%	OR	95% CI
BKPyV (VP1)	338	(91.4)	281	(78.3)	**0.34**	**(0.22-0.53)**	**<0.001**	158	(77.5)	**0.32**	**(0.20-0.53)**	55	(76.4)	**0.31**	**(0.16-0.60)**	*0.755*	61	(83.6)	**0.48**	**(0.23-0.99)**
JCPyV (VP1)	255	(68.9)	207	(57.7)	**0.61**	**(0.45-0.83)**	**0.002**	115	(56.4)	**0.58**	**(0.41-0.83)**	34	(47.2)	**0.40**	**(0.24-0.68)**	*0.160*	52	(71.2)	1.09	(0.62-1.92)
LPyV (VP1)	162	(43.8)	104	(29.0)	**0.51**	**(0.37-0.70)**	**<0.001**	62	(30.4)	**0.53**	**(0.37-0.77)**	22	(30.6)	0.58	(0.33-1.00)	*0.998*	18	(24.7)	**0.43**	**(0.24-0.77)**
KIPyV (VP1)	329	(88.9)	271	(75.5)	**0.38**	**(0.26-0.57)**	**<0.001**	157	(77.0)	**0.41**	**(0.26-0.66)**	53	(73.6)	**0.35**	**(0.19-0.65)**	*0.573*	54	(74.0)	**0.36**	**(0.19-0.67)**
WUPyV (VP1)	365	(98.7)	337	(93.9)	**0.21**	**(0.08-0.55)**	**0.002**	196	(96.1)	0.32	(0.10-1.02)	69	(95.8)	0.34	(0.08-1.46)	*0.823*	63	(86.3)	**0.08**	**(0.03-0.26)**
HPyV-6 (VP1)	345	(93.2)	320	(89.1)	**0.59**	**(0.34-0.99)**	**0.047**	181	(88.7)	0.55	(0.30-1.00)	61	(84.7)	**0.41**	**(0.19-0.88)**	*0.461*	69	(94.5)	1.27	(0.42-3.82)
HPyV-7 (VP1)	297	(80.3)	256	(71.3)	**0.60**	**(0.43-0.85)**	**0.004**	141	(69.1)	**0.54**	**(0.36-0.80)**	53	(73.6)	0.69	(0.38-1.25)	*0.442*	54	(74.0)	0.68	(0.37-1.23)
TSPyV (VP1)	283	(76.5)	249	(69.4)	**0.70**	**(0.50-0.97)**	**0.033**	89	(43.6)	0.70	(0.48-1.03)	48	(66.7)	0.63	(0.36-1.09)	*0.530*	55	(75.3)	0.88	(0.48-1.60)
MCPyV (VP1)	310	(83.8)	289	(80.5)	0.79	(0.54-1.16)	0.230	166	(81.4)	0.79	(0.50-1.24)	56	(77.8)	0.70	(0.38-1.32)	*0.922*	59	(80.8)	0.91	(0.47-1.76)
JCPyV (LT)	26	(7.0)	19	(5.3)	0.74	(0.40-1.36)	0.334	8	(3.9)	0.54	(0.24-1.22)	3	(4.2)	0.57	(0.17-1.96)	*0.921*	8	(11.0)	1.61	(0.68-3.79)

Association estimated by unconditional[a] and multinomial[b] logistic regression adjusted by sex, age (quartiles) and region

[c] p-value for heterogeneity between CLL rai 0 and CLL rai I-IV using case-case analysis

[d] Treated cases with rai I-IV who have received CLL treatment before sample extraction

Figure 1. Box-plot distribution of all polyomaviruses seroreactivity against VP1 antigen, by categorized cases



Viral seroreactivity is provided stratified into controls (A) and disease stage by treatment: (B) CLL rai 0, (C) CLL rai I-IV untreated and (D) CLL rai I-IV treated. Dotted lines represent specific cut-off values for each virus seroprevalence

Supplemental Table 1. Descriptive characteristics of viral capsid seroprevalence in control population

	Total	BKPyV positive n	%	JCPyV positive n	%	LPyV positive n	%	KIPyV positive n	%	WUPyV positive n	%	HPyV-6 positive n	%	HPyV-7 positive n	%	TSPyV positive n	%	MCPyV positive n	%
Control population	370	338	(91.4)	255	(68.9)	162	(43.8)	329	(88.9)	365	(98.6)	345	(93.2)	297	(80.3)	283	(76.5)	310	(83.8)
Sex																			
Men	230	215	(93.5)	165	(71.7)	104	(45.2)	202	(87.8)	227	(98.7)	216	(93.9)	187	(81.3)	179	(77.8)	197	(85.7)
Women	140	123	(87.9)	90	(64.3)	58	(41.4)	127	(90.7)	138	(98.6)	129	(92.1)	110	(78.6)	104	(74.3)	113	(80.7)
		p=0.085		*p=0.164*		*p=0.517*		*p=0.495*		*p=1.000*		*p=0.527*		*p=0.590*		*p=0.450*		*p=0.245*	
Center of recruitment																			
Barcelona	285	262	(91.9)	193	(67.7)	135	(47.4)	250	(87.7)	283	(99.3)	266	(93.3)	235	(82.5)	216	(75.8)	241	(84.6)
Asturias	48	43	(89.6)	36	(75.0)	11	(22.9)	45	(93.8)	47	(97.9)	46	(95.8)	33	(68.8)	39	(81.3)	38	(79.2)
Cantabria	19	17	(89.5)	12	(63.2)	8	(42.1)	14	(73.7)	18	(94.7)	16	(84.2)	14	(73.7)	16	(84.2)	17	(89.5)
Granada	18	16	(88.9)	14	(77.8)	8	(44.4)	16	(88.9)	17	(94.4)	17	(94.4)	15	(83.3)	12	(66.7)	14	(77.8)
		p=0.777		*p=0.615*		***p=0.015***		*p=0.141*		*p=0.061*		*p=0.345*		*p=0.139*		*p=0.548*		*p=0.572*	
Age																			
40-61	90	81	(90.0)	53	(58.9)	30	(33.3)	77	(85.6)	88	(97.8)	83	(92.2)	71	(78.9)	60	(66.7)	73	(81.1)
62-67	83	75	(90.4)	60	(72.3)	39	(47.0)	77	(92.8)	82	(98.8)	75	(90.4)	64	(77.1)	73	(88.0)	66	(79.5)
68-74	95	85	(89.5)	67	(70.5)	41	(43.2)	80	(84.2)	95	(100)	90	(94.7)	81	(85.3)	76	(80.0)	82	(86.3)
75-79	102	97	(95.1)	75	(73.5)	52	(51.0)	95	(93.1)	100	(98.0)	97	(95.1)	81	(79.4)	74	(72.5)	89	(87.3)
		p=0.434		*p=0.133*		*p=0.088*		*p=0.101*		*p=0.626*		*p=0.550*		*p=0.525*		***p=0.005***		*p=0.403*	
trend		*p=0.250*		***p=0.049***		***p=0.030***		*p=0.297*		*p=0.744*		*p=0.266*		*p=0.625*		*p=0.654*		*p=0.140*	
Level of studies*																			
Less than primary	114	107	(93.9)	81	(71.1)	49	(43.0)	101	(88.6)	114	(100)	108	(94.7)	93	(81.6)	94	(82.5)	94	(82.5)
Primary	100	88	(88.0)	69	(69.0)	51	(51.0)	89	(89.0)	97	(97.0)	89	(89.0)	83	(83.0)	73	(73.0)	84	(84.0)
Secondary	98	90	(91.8)	70	(71.4)	38	(38.8)	87	(88.8)	96	(98.0)	93	(94.9)	78	(79.6)	73	(74.5)	88	(89.8)
University	58	53	(91.4)	35	(60.3)	24	(41.4)	52	(89.7)	58	(100)	55	(94.8)	43	(74.1)	43	(74.1)	44	(75.9)
		p=0.514		*p=0.484*		*p=0.356*		*p=1.000*		*p=0.144*		*p=0.349*		*p=0.569*		*p=0.325*		*p=0.137*	
trend		*p=0.682*		*p=0.294*		*p=0.503*		*p=0.866*		*p=0.784*		*p=0.732*		*p=0.255*		*p=0.186*		*p=0.813*	
Previous cancer																			
None	327	298	(91.1)	220	(67.3)	143	(43.7)	288	(88.1)	322	(98.5)	303	(92.7)	263	(80.4)	249	(76.1)	272	(83.2)
Yes	41	39	(95.1)	33	(80.5)	19	(46.3)	40	(97.6)	41	(100)	40	(97.6)	33	(80.5)	32	(78.0)	36	(87.8)
		p=0.555		p=0.107		p=0.868		p=0.104		p=1.000		p=0.337		p=1.000		p=1.000		p=0.653	
Family history of cancer																			
None	83	74	(89.2)	59	(71.1)	36	(43.4)	76	(91.6)	83	(100)	78	(94.0)	66	(79.5)	65	(78.3)	68	(81.9)
Non-hematotological	221	204	(92.3)	153	(69.2)	96	(43.4)	196	(88.7)	216	(97.7)	205	(92.8)	176	(79.6)	168	(76.0)	189	(85.5)
Hematological cancer	27	24	(88.9)	16	(59.3)	11	(40.7)	23	(85.2)	27	(100)	24	(88.9)	20	(74.1)	21	(77.8)	19	(70.4)
		p=0.513		*p=0.505*		*p=0.984*		*p=0.579*		*p=0.562*		*p=0.617*		*p=0.764*		*p=0.955*		*p=0.121*	
Smoking status																			
Never Smoker	173	159	(91.9)	113	(65.3)	84	(48.6)	157	(90.8)	171	(98.8)	159	(91.9)	139	(80.3)	134	(77.5)	143	(82.7)
Current smoker	59	52	(88.1)	41	(69.5)	27	(45.8)	52	(88.1)	57	(96.6)	56	(94.9)	42	(71.2)	46	(78.0)	51	(86.4)
Ex-smoker	136	125	(91.9)	100	(73.5)	50	(36.8)	119	(87.5)	135	(99.3)	128	(94.1)	114	(83.8)	102	(75.0)	115	(84.6)
		p=0.657		*p=0.299*		*p=0.108*		*p=0.638*		*p=0.309*		*p=0.697*		*p=0.128*		*p=0.867*		*p=0.796*	

Unless otherwise specified, provided p-values have been estimated using Fisher exact heterogeneity test. P-values for trend estimated using unconditional logistic regression

Supplemental Table 2. Association between polyomavirus seroreactivity and CLL; all cases and stratified by disease stage

				Stratification by rai stage																
Viral capsid		**Controls**		**All cases[a] (n=359)**					**CLL Rai 0 (n=204)**					**CLL Rai I-IV (n=72)**					**Treated[c] (n=73)**				
seroreactivity for		**N**	**%**	**N**	**%**	**OR**	**95% CI**	***trend***	**N**	**%**	**OR**	**95% CI**	***trend***	**N**	**%**	**OR**	**95% CI**	***trend***	**N**	**%**	**OR**	**95% CI**	***trend***
BKV	**1st tertile**	112	(33.1)	114	(40.6)				59	(37.3)				30	(54.5)				21	(34.4)			
	2nd tertile	114	(33.7)	106	(37.7)	0.91	(0.63-1.33)		62	(39.2)	0.99	(0.50-1.44)		14	(25.5)	**0.46**	**(0.23-0.93)**		27	(44.3)	1.40	(0.73-2.69)	
	3rd tertile	112	(33.1)	61	(21.7)	**0.53**	**(0.35-0.81)**	***0.004***	37	(23.4)	0.59	(0.35-1.09)	**0.044**	11	(20.0)	**0.38**	**(0.18-0.80)**	**0.005**	13	(21.3)	0.69	(0.32-1.47)	0.394
JCV	**1st tertile**	84	(32.9)	94	(45.4)				49	(42.6)				16	(47.1)				25	(48.1)			
	2nd tertile	86	(33.7)	76	(36.7)	0.79	(0.51-1.21)		39	(33.9)	0.77	(0.46-1.29)		13	(38.2)	0.80	(0.36-1.78)		22	(42.3)	0.87	(0.45-1.68)	
	3rd tertile	85	(33.3)	37	(17.9)	**0.39**	**(0.24-0.63)**	***<0.001***	27	(23.5)	**0.53**	**(0.30-0.93)**	**0.024**	5	(14.7)	**0.32**	**(0.11-0.91)**	**0.033**	5	(9.6)	**0.21**	**(0.08-0.58)**	**0.003**
LPV	**1st tertile**	55	(34.0)	51	(49.0)				31	(50.0)				12	(54.5)				8	(44.4)			
	2nd tertile	53	(32.7)	34	(32.7)	0.71	(0.40-1.27)		18	(29.0)	0.63	(0.31-1.28)		8	(36.4)	0.68	(0.25-1.85)		7	(38.9)	0.92	(0.30-2.77)	
	3rd tertile	54	(33.3)	19	(18.3)	**0.37**	**(0.19-0.70)**	***0.003***	13	(21.0)	**0.40**	**(0.19-0.86)**	**0.016**	2	(9.1)	**0.16**	**(0.03-0.77)**	**0.017**	3	(16.7)	0.41	(0.10-1.65)	0.227
KIV	**1st tertile**	109	(33.1)	149	(55.0)				75	(47.8)				33	(62.3)				34	(63.0)			
	2nd tertile	112	(34.0)	75	(27.7)	**0.48**	**(0.33-0.71)**		52	(33.1)	0.65	(0.42-1.02)		10	(18.9)	**0.30**	**(0.14-0.63)**		13	(24.1)	**0.39**	**(0.19-0.78)**	
	3rd tertile	108	(32.8)	47	(17.3)	**0.31**	**(0.21-0.48)**	***<0.001***	30	(19.1)	**0.37**	**(0.22-0.61)**	**<0.001**	10	(18.9)	**0.32**	**(0.15-0.69)**	**0.001**	7	(13.0)	**0.24**	**(0.10-0.58)**	**<0.001**
WUV	**1st tertile**	121	(33.2)	188	(55.8)				106	(54.1)				40	(58.0)				39	(54.2)			
	2nd tertile	125	(34.2)	94	(27.9)	**0.48**	**(0.34-0.69)**		55	(28.1)	**0.50**	**(0.33-0.76)**		15	(21.7)	**0.36**	**(0.19-0.68)**		29	(40.3)	**0.49**	**(0.27-0.91)**	
	3rd tertile	119	(32.6)	55	(16.3)	**0.29**	**(0.20-0.44)**	***<0.001***	35	(17.9)	**0.33**	**(0.21-0.52)**	**<0.001**	14	(20.3)	**0.35**	**(0.18-0.67)**	**0.001**	4	(5.6)	**0.11**	**(0.04-0.31)**	**<0.001**
HPyV-6	**1st tertile**	114	(33.0)	155	(48.7)				82	(45.6)				24	(39.3)				42	(61.8)			
	2nd tertile	117	(33.9)	88	(27.7)	**0.55**	**(0.38-0.80)**		51	(28.3)	**0.61**	**(0.39-0.94)**		23	(37.7)	0.92	(0.49-1.73)		13	(19.1)	**0.30**	**(0.15-0.59)**	
	3rd tertile	114	(33.0)	75	(23.6)	**0.47**	**(0.32-0.69)**	***<0.001***	47	(26.1)	**0.58**	**(0.37-0.92)**	**0.015**	14	(23.0)	0.55	(0.27-1.14)	0.121	13	(19.1)	**0.28**	**(0.14-0.56)**	**<0.001**
HPyV-7	**1st tertile**	99	(33.3)	129	(50.4)				68	(48.2)				31	(58.5)				25	(46.3)			
	2nd tertile	98	(33.0)	81	(31.6)	**0.62**	**(0.42-0.93)**		48	(34.0)	0.69	(0.43-1.10)		12	(22.6)	**0.40**	**(0.19-0.83)**		20	(37.0)	0.80	(0.41-1.56)	
	3rd tertile	100	(33.7)	46	(18.0)	**0.35**	**(0.22-0.54)**	***<0.001***	25	(17.7)	**0.36**	**(0.21-0.61)**	**<0.001**	10	(18.9)	**0.31**	**(0.15-0.68)**	**0.001**	9	(16.7)	**0.36**	**(0.16-0.82)**	**0.017**
TSV	**1st tertile**	96	(33.9)	109	(43.8)				58	(41.1)				19	(39.6)				29	(52.7)			
	2nd tertile	93	(32.9)	95	(38.2)	0.89	(0.60-1.33)		52	(36.9)	0.92	(0.57-1.48)		21	(43.8)	1.10	(0.55-2.20)		20	(36.4)	0.70	(0.36-1.34)	
	3rd tertile	94	(33.2)	45	(18.1)	**0.42**	**(0.26-0.66)**	***<0.001***	31	(22.0)	**0.55**	**(0.32-0.93)**	**0.029**	8	(16.7)	**0.41**	**(0.17-0.99)**	0.061	6	(10.9)	**0.21**	**(0.08-0.53)**	**0.001**
MCV	**1st tertile**	105	(33.9)	123	(42.6)				70	(42.2)				23	(41.1)				26	(44.1)			
	2nd tertile	101	(32.6)	109	(37.7)	0.92	(0.63-1.34)		59	(35.5)	0.83	(0.53-1.30)		22	(39.3)	0.99	(0.52-1.90)		24	(40.7)	1.04	(0.55-1.94)	
	3rd tertile	104	(33.5)	57	(19.7)	**0.46**	**(0.30-0.70)**	***<0.001***	37	(22.3)	**0.50**	**(0.30-0.81)**	**0.005**	11	(19.6)	0.47	(0.22-1.03)	0.066	9	(15.3)	**0.39**	**(0.17-0.87)**	**0.031**

Association estimated by unconditional[a] and multinomial[b] logistic regression adjusted by sex, age (quartiles) and region

[c] Treated cases with rai I-IV who have received CLL treatment before sample extraction

6. DISCUSSION

In the three case-control studies conducted, we identified associations between MCPyV and diffuse large b-cell lymphoma as well as between BKPyV and MCPyV with bladder cancer, which initially suggest a potential carcinogenic role of these polyomaviruses. By contrast, an inverse association was observed for the nine polyomaviruses tested in CLL, probably due to an immune impairment. These initial interpretations are further discussed and developed in this section by providing an overall view on the potential roles of polyomaviruses across the different malignancies studied and evaluating our results in terms of their strengths and limitations. Further comments on the interpretation of the results in relation to the controversial use of serology as a biomarker of exposure, and in the probable immune impairment observed in CLL are provided.

Details on specific aspects of each of the malignancies explored in this thesis are provided in each of the papers in Results section.

6.1 General discussion

a) Potential carcinogenic role of polyomaviruses

Merkel cell polyomavirus, diffuse large b-cell lymphoma and chronic lymphocytic leukemia

The Epilymph study data showed a significant increased risk of DLBCL (OR=6.10; 95%CI=1.88-19.75) among MCPyV seroprevalent subjects. When the same DLBCL samples and matched controls were tested for BKPyV and JCPyV seroprevalence, null associations were obtained. Regarding MCPyV seroreactivity, using optical density units, no median differences between DLBCL and controls were detected but a borderline significant median difference was observed when measured by means of endpoint titration (374.2 EIA units in DLBCL versus 232.6 EIA units in controls). The increased risk of DLBCL among MCPyV seroprevalent subjects was replicated when seroreactivity was measured by fluorescent bead-based multiplex serology.

The increased MCPyV seroprevalence in DLBCL subjects is in agreement with the increased seroprevalence observed for Merkel cell carcinoma subjects[123–125]. Our results lack the association with increased seroreactivity, but also Carter et al.[124] did not observe median seroreactivity differences between Merkel cell carcinoma subjects and controls. Regarding molecular data in tumor tissue, two previous case-series studies reported low MCPyV DNA detection in DLBCL samples[155,157], in agreement with our lack of MCPyV DNA in five DLBCL biopsies but, in contrast to the higher MCPyV seroprevalence. Nevertheless, the high seroprevalence could be the result of a non-carcinogenic viral reactivation because of a DLBCL-related immunosuppression.

Because of the ubiquitous presence of MCPyV, Moore and Chang[189] argue on quantity rather than presence as an indicator of a potential causal association when studying common infections. Based on the molecular characteristics observed in Merkel cell carcinoma subjects, they suggest an event-dependent causal model in which the risk of developing the disease increases by acquiring the infection (common), losing immune surveillance (uncommon) and undergoing viral specific mutations (rare). Therefore, this model leans towards a non-carcinogenic association of MCPyV with DLBCL.

Since the publication of our paper, no further related data has been published for MCPyV, but for JCPyV and BKPyV in DLBCL. Tseng et al.[190] detected 9 JCPyV positive and 5 BKPyV positive in 16 DLBCL tissue samples of the gastrointestinal tract, in contrast to only 1 BKPyV positive out of 20 gastrointestinal control samples. These results contrast with our null associations for JCPyV and BKPyV seroprevalence as well as those observed by Rollison et al.[148], who did not observe any increased risk of subsequent DLBCL among seroprevalent subjects using pre-diagnostic samples. Engels et al.[154] also obtained a null association for BKPyV seroprevalence although a significant decreased risk of DLBCL among JCPyV seroprevalent subjects. The detection of these polyomaviruses in DLBCL samples by Tseng et al. could indicate a higher presence, not necessarily carcinogenic but latent or persistent, in lymphocytes (DLBCL main origin cell type) rather than in epithelial cells (main cell type in gastrointestinal control samples).

Regarding CLL and MCPyV, opposed directions of association were obtained between studies; the Epilymph study data suggested an increasing risk (OR=1.49; 95%CI=0.80-2.77) whereas the MCC-Spain study data suggested a decreasing one (OR=0.79; 95%CI=0.54-1.16). These apparently contradictive results are explained by the different age distribution in control population. Age was not associated with MCPyV seroprevalence in the MCC-Spain study, but significant differences were observed in the Epilymph study, probably because of a wider age range in control population. In the Epilymph study, all control population had an 80% MCPyV seropositivity in comparison to an 86% in CLL. However, when we randomly select one control for each CLL case by sex, age and center, we obtain a MCPyV seroprevalence of 88% in controls. Restriction of control population to the age range at which CLL cases are diagnosed (older ages) did not change direction or magnitude of association in original analyses and therefore the use of a restricted control population was discarded because of a potential data stretching.

Paulson et al.[123] measured seroreactivity against MCPyV VP1, LT-Ag and sT-Ag in Merkel cell carcinoma cases and in controls, similar to what we did in the MCC-Spain study. In this study, VP1 seroprevalence was 66% in controls versus 91% in Merkel cell carcinoma cases ($p<0.001$), but only around 1% of controls were seropositive for LT-Ag and sT-Ag in contrast to 26% and 40% respectively, for cases. Therefore, the very low seroprevalence among cases of MCPyV LT-Ag and MCPyV sT-Ag (<0.5%) in the MCC-Spain study, is similar to that of controls in the study of Paulson whereas the higher seroprevalence against MCPyV VP1 (80%) is likely reflecting the older control population in the MCC-Spain study.

Our findings on MCPyV in CLL likely reflect a null association. This interpretation differs with the encouraging results from those who detected a mutated sequence of MCPyV LT-Ag in the nucleus of CLL cells[170,171]. However, only an 8% (6 out of 70) of the total CLL samples, previously processed to contain mainly CD19+/CD5+ CLL cells, showed this mutation. No further studies on this truncated LT-Ag in CLL cells have been published to date, and therefore replication data are missing. On the other hand, the increased incidence of Merkel cell carcinoma in CLL cohorts, and

vice versa[100,165,166], might be explained by shared immunosuppression instead of a shared etiologic pathway.

BK polyomavirus, Merkel cell polyomavirus and bladder cancer

The Epicuro study data showed an increased risk of bladder cancer among seroprevalent subjects with high BKPyV (OR=1.37; 95%CI=1.04-1.80) and high MCPyV (OR=1.48; 95%CI=1.16-1.88) seroreactivity but a null association for JCPyV seroreactivity.

Our data contrast with the null association for BKPyV obtained by Newton et al.[179], who used prediagnostic serum samples from the European Prospective Investigation into Cancer and Nutrition study (a.k.a EPIC study) although based on only 9 bladder cancer cases within the cohort. As per BKPyV DNA in tissue sample, the detection rate in previous studies ranges between 5% in the USA[191] to around 50% in Italy[177,178]. A recent paper by Bulut et al.[192] did not observe differences in prevalence of BKPyV DNA or mRNA but detected a higher copy number of BKPyV mRNA in bladder cancer subjects compared to controls. This study might suggest an increased BKPyV expression, in agreement with an active infection.

As per MCPyV seroreactivity and bladder cancer, Loyo et al.[181] obtained a high MCPyV DNA positivity (75%) although based on only 8 samples. Polesel et al.[180] tested for several polyomaviruses DNA prevalence among bladder cancer subjects and controls, including BKPyV and MCPyV, and did not observe any association but used viruria as exposure biomarker, which has raised concerns about its appropriateness[128].

An increased seroreactivity of BKPyV and MCPyV agrees with the increased seroreactivity observed for MCPyV in Merkel cell carcinoma subjects[44,121,125,126] although it lacks the association with viral seroprevalence. Viscidi et al.[44] also reported a lack of association with MCPyV seroprevalence in Merkel cell carcinoma subjects, after adjustment by age. The same laboratory was used in both studies, which might suggest that using this methodology, an association with seroprevalence might not be measurable although data in Merkel cell carcinoma subjects is based on a small sample size. Nevertheless, if a high seroreactivity is assumed to correlate with viral quantity, the results in bladder cancer agree with the

suggested model from Moore and Chang[189] on carcinogenicity of polyomaviruses.

b) Serology data: Exposure biomarker vs. potential reverse causality

The use of serology in epidemiological studies evaluating causal relationships raises two main concerns. On one hand, immune response against viral antigens is an indirect method of quantifying viral exposure, whereas on the other hand, the use of serum samples at diagnosis complicates the interpretation of results because of a potential reverse causality effect.

Under normal circumstances, immune response by antibodies occurs as follows[193]. After an initial contact with an infectious agent, there is an early IgM response that rapidly decreases, followed by a higher and prolonged IgG response. At a second contact with the infectious agent, IgG would be the initial responder boosting a higher response than the original one, as observed in Figure 7.

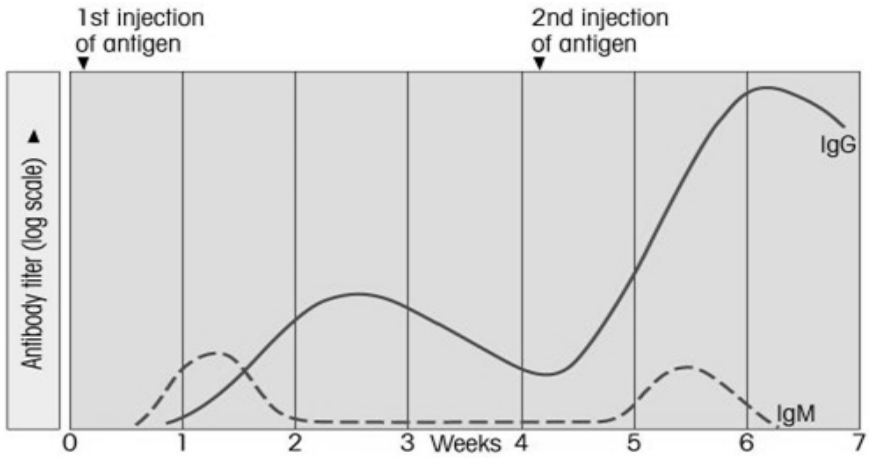


Figure 7. IgM and IgG antibody responses at a primary and secondary contact with antigen.
(From Roitt's essential immunology book[193])

Therefore, in a latent or persistent infection, such as suspected for polyomaviruses in humans, a viral reactivation may behave as a second contact to antigen boosting a higher seroreactivity. However, because of the scarce data on polyomavirus infection

natural history and on the lifetime interaction between the host immune system and polyomaviruses, other explanations should be taken into account. A high seroreactivity could reflect an initial infection that generated a high seroreactivity and that afterwards has remained high or that has been slowly increasing over time. Additionally, because of the ubiquitous presence of polyomaviruses in the environment, we could also be measuring a recent innocuous polyomavirus reinfection.

Findings from previous studies suggest that the actual model might be far more complex. Longitudinal data for 25 years from 17 subjects within the Multicenter AIDS Cohort Study (a.k.a MACS study) showed two patterns of IgG levels after seroconversion into MCPyV seropositivity[194]. 11 patients showed a gradual increase in IgG levels whereas 6 subjects converted into seronegative after 1-2 years (see Figure 8). However, these data must be interpreted cautiously because of missing details on HIV/AIDS status and other potential differences between these pattern subjects.

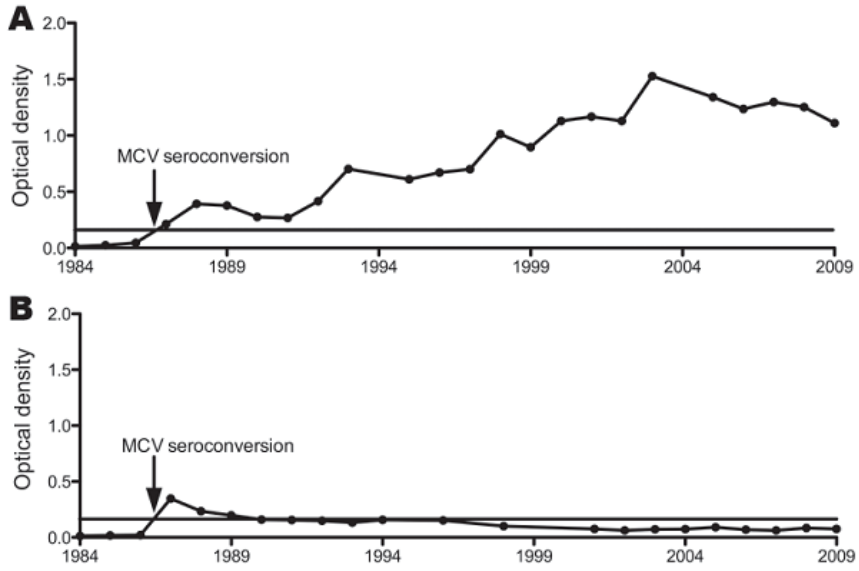


Figure 8. Different patterns of IgG levels behavior over time within the MACS study.
(From Tolstov et al.[194])

Seroconversion into seronegative, or seroreactivity values under cutoff value among previously infected subjects, was also evidenced by Kumar et al. who observed that some IgG seronegative subjects show T-cell immunity against MCPyV. This fact suggests a lack of persistence in IgG immune response among subjects previously exposed to an MCPyV infection, or cross-reactivity of T-cell epitopes with polyomaviruses other than MCPyV and TSPyV[195,196].

Additionally, because of the sample extraction being taken at disease diagnosis, a reverse causality effect cannot be discarded. Using retrospective data, it is not possible to know whether the reactivation took place prior or after the cancer onset, and it is therefore impossible to confirm if we are measuring, respectively, an infectious reactivation that causes cancer or a non-carcinogenic viral reactivation due to cancer-related immunosuppression. However, several observations can help interpreting the serological patterns observed in cases and controls and provide some guidance on its possible interpretation.

In a reverse causality setting, at increasing disease stage, a loss of viral immunosurveillance would be expected, leading to a higher probability of viral reactivation. In this thesis, neither DLBCL nor bladder cancer showed differences in seroprevalence or seroreactivity across disease stages.

Another indicator would be the identification of discrepancies in the immune response between polyomaviruses under a similar immune setting. In the same substudy population from the Epilymph study, DLBCL subjects showed an OR=7 for MCPyV seroprevalence, whereas JCPyV and BKPyV showed flat results for viral seroprevalence. A discordant observation was also observed for BKPyV and MCPyV seroreactivity in comparison to JCPyV seroreactivity in bladder cancer, the latter showing a null association. These differences in association might reflect different polyomaviruses immunogenicity in latent innocuous infections or viral reactivation.

Finally, the same concerns raised in this thesis are applicable to previously established associations between infections and cancer (see Table 1) when measured by means of seroresponse.

Nevertheless, these associations, including MCPyV and Merkel cell carcinoma, have shown significant increased seroprevalences than control population in case-control studies (see Table 5).

Table 5. Observed significant risk range for seroprevalence in confirmed associations between infections and cancer

Infection	*Pathology associated*	*Risk range*[a]
MCPyV	Merkel cell carcinoma	OR (VP1) =5 OR (LT-Ag) =17 OR (sT-Ag) =63
HTLV-1	Adult t-cell leukemia	OR=3-5[b]
HBV	Hepatocellular cancer	OR=5-50
HBC	Hepatocellular cancer	OR=4-60
HHV-8	Kaposi's sarcoma	OR=2-25[c]
H. pylori	Non-cardia gastric cancer	OR=2-9

[a] Risk range provides risks obtained by case-control/cross-sectional studies (OR) among those with significant results and using more robust data[1,128,174,197–199]
[b] Potentially underestimated due to viral seroprevalence required for diagnosis
[c] Results highly dependent on HIV status

c) CLL-related immunosuppression

In CLL cases within the MCC-Spain study, unexpected lower seroreactivities and seroprevalences for the 9 polyomaviruses tested in comparison to controls were observed.

These observations are more likely to reflect a reverse causality effect due to immune impairment among cases rather than being interpreted as a lower proportion of infected cases than controls. If CLL decreases IgG levels, it is plausible that, as observed in pattern B from Figure 8, more cases than controls may have seroreactivity values under a defined cut-off point for positivity. This would result in a lower sensitivity for the chosen cut-off point and lead to an increase in false negative subjects.

CLL subjects have been previously reported to show hypogammaglobulinemia (i.e low IgG levels) before disease onset,

and neutropenia and cell-mediated immunity defects at increasing disease stage and after treatment[200,201].

CLL is a slow progressing disease, completely asymptomatic at early stages, usually diagnosed by chance. Furthermore, up to 77 months prior to its diagnosis, clonal b-cells can already be identified in blood samples[202]. These facts suggest that the disease, and related malfunctions and deficiencies, might be present several years prior to clinical diagnosis. Therefore, the increased incidences of CLL and Merkel cell carcinoma as second cancer in relation to each other[100,165,166], could be the result of an undiagnosed CLL-related immunosuppression that reactivates MCPyV. Depending on the timing of CLL diagnosis, it could incorrectly seem that CLL is the second cancer rather than the primary tumor.

On the other hand, the use of serology as exposure biomarker within the MCC-Spain study in CLL subjects might seem inappropriate and inconclusive. About its appropriateness, previous studies on infections seroprevalence within the Epilymph study did not suggest an immune impairment. Excluding subjects with HIV and/or organ-transplant recipients, non-significant although increased risks of CLL were obtained for seroprevalence against HCV (OR=1.47; 95%CI=0.59–3.67)[203] and seroprevalence against HHV-8 (OR=1.16; 95%CI=0.48 – 2.79)[204]. Seroprevalence against *H. pylori* showed a decreasing risk of CLL (OR=0.71; 95%CI=0.45-1.12)[205], but since most lymphomas tested in this analysis showed similar values except gastric lymphomas and splenic marginal zone lymphomas, decreased risks were interpreted as a lack of association. Based on our findings on polyomaviruses, the use of serology from a quantitative approach is not recommended to study CLL once the disease has developed, but it can still be used with a qualitative approach (such as the aberrant pattern of immune response against EBV[206]) or in prospective studies (although several years in advance to avoid any underlying immunosuppressing effect). Regarding the inconclusive results, it is relevant to show that in the MCC-Spain study, CLL subjects at Rai I-IV stage who underwent treatment, showed higher JCPyV VP1 and LT-Ag seroprevalences than untreated subjects and controls. Interestingly, JCPyV causes progressive multifocal leukoencephalopathy, an opportunistic disease with an increased incidence in CLL subjects[207]. This suggests that even under immune impairment

conditions, JCPyV can be reactivated and induce a substantial increase in seroreactivity of VP1 and LT-Ag. Therefore, the almost absolute absence of antibodies against MCPyV LT-Ag and sT-Ag in CLL patients, irrespective of stage at diagnosis, is consistent with a null association for MCPyV. Nevertheless, since little is known about the other polyomaviruses immunogenicity, no conclusions can be reached on their role in CLL, but if these viruses can be reactivated as JCPyV apparently does, null associations are hypothesized.

Regarding the under-detection of actually infected subjects in CLL subjects, an opposed effect cannot be ruled out in DLBCL, an AIDS-related lymphoma. DLBCL incidence is higher under immunosuppressing conditions, which also enhances polyomavirus reactivation. Therefore, it is possible that the common initial immunosuppression explains the association observed between MCPyV and DLBCL; an increasing seroreactivity such as the one observed in pattern A of Figure 8 could change low values categorization from seronegative to seropositive. Therefore, control population in the Epilymph study would be showing the usual rate of false seronegative subjects observed by Kumar et al.[195] whereas DLBCL subjects might show all truly infected patients rather than a higher infection rate. If this increasing seroreactivity could reflect a viral reactivation with a pathogenic or a carcinogenic role remains unknown.

6.2 Strengths and limitations

When compared to previously published studies, one of the main strengths of this thesis is the use of solid epidemiologic case-control studies instead of case series without or with only a few control subjects. Additionally, the studies used in this thesis contain the largest number of Spanish subjects tested to date for polyomaviruses in bladder cancer and lymphoproliferative disorders. The comparison with adequate controls allows establishing robust associations between exposure and outcome, even with infections such as polyomaviruses that are almost ubiquitous. However, because of the retrospective design, temporal associations cannot be evaluated.

Because of the inclusion criteria, most of the subjects in lymphoma and bladder cancer studies were incident and therefore, most of them had not been treated when recruited and/or when the sample was taken. Nevertheless, data on treatment was always available, so that sensitivity analyses by exclusion of treated subjects were possible. Furthermore, whenever data was available, organ transplanted or HIV positive subjects, because of their related immunosuppression, were excluded from the analysis.

The use of biomarkers is a useful tool to measure exposure and avoid misclassification, especially in asymptomatic initial infections, such as those caused by polyomaviruses but its use as a biomarker of the potential carcinogenic effect of polyomaviruses may also be, as described before, controversial. This effect might be more relevant than originally suspected in lymphoproliferative disorders, a disease within the immune system. Nevertheless, it can provide useful information in bladder cancer, in which numerous studies have examined auto-reported infections through questionnaires leading to inconclusive results.

In the MCC-Spain study, population controls were used while hospital-based controls were used in the lymphomas and bladder cancer studies. In the latter studies, it is unlikely that any of the reasons for hospitalization (that were mainly trauma and minor surgery related) could have had an impact in the associations observed. Furthermore, if any, it would likely cause a false increase in seroreactivity which would suggest an underestimation in the risks obtained rather than a chance finding.

Furthermore, although two different techniques were used to measure viral seroreactivity, we could compare them. By using a correlation analysis, we confirmed that both measurements were correlated in samples from the same subjects. Additionally, when association analyses were repeated using multiplex serology data instead of EIA for DLBCL and MCPyV, a significant increased seroprevalence was obtained, which allowed replication of our original results.

6.3 Novelty of results and future perspectives

This thesis provides novel data on polyomaviruses carcinogenicity potential. To date, using serology data, only BKPyV and JCPyV have been studied in lymphoproliferative disorders, whereas we have looked for MCPyV in 11 lymphoma subtypes and for up to nine polyomaviruses in chronic lymphocytic leukemia. As per bladder cancer, the previous study only tested for BKPyV in a limited number of cases whereas we tested for three polyomaviruses in over a thousand cases.

The strong host immune deregulation observed in CLL patients, potentially resulting in an inability to amount a proper adaptive immune response against common infections like polyomaviruses contrasts with the increased seroresponse observed in DLBCL, a hematological malignancy as CLL. The reason for these antagonistic seroresponses remains uncertain, but the similar increased seroresponse association observed for bladder cancer opens the door to verify the consistency of our results in other settings and with other methodological approaches.

Retrospective data are further more relevant when a null association is observed than a significant one; a null association is more likely to reflect a truly lack of association than a positive association to reflect a truly causative role. Therefore, although our data are significantly associated with cancer, the associations observed are not as strong as we would expect in a causal relation and further studies are required to confirm our suspected lack of association.

From an epidemiological perspective, the use of prospective studies (i.e cohort studies like EPIC) could provide information on the biological time line of these associations. If serum samples are retrieved over a decade before the disease is diagnosed, any potential immunosuppressing disease effect over the host immune response against infections would be likely avoided. A strong seroreactivity, measured preceding the disease diagnosis, could then be more accurately identifying a possible carcinogenesis mechanism. Furthermore, the combined use of serological biomarkers, relatively accessible nowadays, and existing cohorts would provide a major contribution in the topic.

Undoubtedly, the verification of these associations by means of molecular biomarkers, such as viral DNA, mRNA or antigen expression in tissue, is necessary for any solid conclusion on our observations on DLBCL and bladder cancer, especially for MCPyV in bladder cancer. Initial efforts to use tissue samples from DLBCL resulted in lack of detection, although the limited number of samples tested requires being cautious in any interpretation. On the other hand, other researchers involved in the Epicuro study have been looking for polyomaviruses in bladder cancer cell lines (unpublished data). Few cell lines showed MCPyV DNA presence at low DNA copy numbers, but cell lines are not fully representatives of bladder cancer tissue and results must also be interpreted cautiously.

7. CONCLUSIONS

- ✓ The sero-epidemiology of nine polyomaviruses in three Spanish studies confirmed the almost ubiquitous seroprevalence of these viruses in the adult Spanish population.

- ✓ Polyomaviruses are unlikely to play a role in lymphomagenesis:

    - Overall, no associations were obtained between tested polyomavirus seroresponse and studied lymphoma subtypes.
    - As an exception, diffuse large b-cell lymphoma was associated with MCPyV seroprevalence; but no association with seroreactivity levels and the lack of MCPyV DNA detection in tumor samples do not support causality.
    - The lack of seroresponse to MCPyV oncoproteins, LT-Ag and sT-Ag, and the low VP1 seroprevalence, are suggestive of an unlikely role of MCPyV in chronic lymphocytic leukemia.

- ✓ The associations of polyomaviruses with bladder cancer needs to be further explored:

    - BKPyV and MCPyV seroreactivities were associated with bladder cancer but the moderate magnitudes cast doubts on a potential clonal response.
    - Others have reported 5-50% for BKPyV DNA presence in tissue but data for MCPyV remains scanty.

Further studies with a prospective design for both pathologies and testing for MCPyV in bladder cancer are required to confirm these conclusions.

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