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**Title:** The trichodysplasia spinulosa-associated polyomavirus: discovery - prevalence - infection - expression  
**Issue Date:** 2016-06-21
Partly based on the following reviews:

**The Trichodysplasia Spinulosa-Associated Polyomavirus: Virological Background And Clinical Implications**

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*APMIS, 2013, 121:770-782*

**From Stockholm To Malawi: Recent Developments In Studying Human Polyomaviruses**

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*Journal of General Virology, 2013, 94:482-496*
Trichodysplasia spinulosa

Clinical description
Trichodysplasia spinulosa (TS) is a rare skin disease first reported by Izakovic and colleagues in 1995. In 1999, Haycox and colleagues fully described the disease and introduced the term ‘trichodysplasia spinulosa’. They showed for the first time the presence of virus particles in TS skin lesions and suggested a viral etiology. Ever since, approximately 30 comparable cases have been published.

TS has been primarily observed in immunosuppressed solid organ transplant patients and in chronic and acute lymphocytic leukemia patients. Recently TS was also described in a patient with Gorlin syndrome (a genetic disorder causing multiple basal cell carcinomas) treated with vismodegib (hedgehog signaling pathway inhibitor), and in a patient with the autoimmune disease lupus erythematosus receiving immunosuppressive drugs. Despite disparity in nomenclature (e.g. trichodysplasia, pilomatrix dysplasia of immune suppression, cyclosporine-induced folliculodystrophy, or viral-associated trichodysplasia), every case is clinically characterized by follicular papules and hyperkeratotic spiny white-yellowish spicules distributed on the face and ears (FIGURE 1A), and to a lesser extent on extremities, trunk and scalp. TS may be accompanied by non-scaring alopecia of the eyebrows, lashes and scalp.

FIGURE 1: CLINICAL MANIFESTATION OF TRICHODYSPLASIA SPINULOSA.
A. Follicular papules, hyperkeratotic spicules and non-scaring alopecia of the eyebrows presented on a 5 year old immunosuppressed girl. B. Histopathology (HE staining) showing a skin cross-section with distended hair follicles.
Histology and viral pathology

Histology of TS skin biopsies shows enlargement of hair follicles and in most cases hyperplasia (acanthosis) of the epidermis. TS follicles are absent of hair shafts, and papilla with abnormal corneocytes are observed that fill the infundibula of the follicles (FIGURE 1B). Sometimes mild perifollicular lymphocytic inflammation is detected. The irregular inner root sheath (IRS) cells of the hair follicle of TS patients show enlarged, dystrophic, prominent eosinophilic, perinuclear globules, probably representing accumulation of trichohyalin. Trichohyalin is a protein that confers mechanical strength to the inner root hair sheath. Strong and frequent staining of the affected IRS cells with the proliferation marker Ki-67 indicates that IRS cells are hyperproliferating.\(^2,3\) Electron microscopy of the lesions reveals the presence of intranuclear, crystalloid-organized, regularly-spaced 38–45-nm virus particles in IRS cells.\(^2,14,22-24,26,29,30,32\) Until 2010, the identity of the virus remained unknown, but the virus capsid size and appearance was known to be consistent with either a papillomavirus or a polyomavirus. Virus culture from fresh TS materials, in an attempt to identify the nature of the virus particles, remained unsuccessful,\(^2,30\) and papilloma- or polyomavirus (degenerated) PCR detection failed.\(^2,22\)

Virus discovery

With the steady advancement of nucleic acid amplification, detection and sequencing techniques, the need for cell culture to detect the presence of a virus was bypassed. Improved/updated versions of these molecular techniques allowed sensitive and high-throughput analysis of large numbers of clinical samples that caused a revolution in virus discovery.

Rolling-circle amplification

A powerful tool for the molecular detection of unknown small, circular, double-stranded DNA genomes, such as papilloma- and polyomaviruses, is a technique called rolling-circle amplification (RCA) (FIGURE 2).\(^33,34\) In nature, replication of circular DNA molecules frequently occurs through a rolling-circle mechanism and RCA mimics this amplification machinery. This technique exploits bacteriophage phi29 (ϕ29) DNA polymerase, a high-fidelity enzyme with 3’ – 5’ exonuclease proof-reading activity. Error rates of ϕ29 DNA polymerase have been calculated to be 9.5 x 10\(^{-6}\).\(^35\) Furthermore, the enzyme has strong strand-displacement properties. In combination with random hexamer primers, ϕ29 DNA polymerase exponentially amplifies circular DNA templates, up to 10\(^7\)-fold.\(^36\) The hexamers randomly anneal to the denatured template DNA (FIGURE 2A) and are extended by the ϕ29 DNA polymerase (FIGURE 2B). When the polymerase reaches the downstream extended primer, strand displacement will occur (FIGURE 2C). Secondary
priming events subsequently take place on the single-stranded displaced strands of the initial RCA step followed by extension (FIGURE 2D). Finally, RCA preferentially produces high-molecular-weight DNA molecules with concatenated (linearized) stretches of DNA which can subsequently be analysed by restriction endonuclease digestion, cloning and sequencing. RCA combined with strategies such as plasmid-safe exonuclease V and NotI restriction endonuclease digestion treatment of DNA can enrich the original sample for viral DNA by reducing the content of host genomic DNA. With the help of RCA human polyomavirus associated with trichodysplasia spinulosa (TSV or TSPyV) could be identified.

**FIGURE 2. ROLLING-CIRCLE AMPLIFICATION.**
A. Annealing of the random hexamer primers (in red) to the circular viral genome (template) DNA. B. Extension by φ29 DNA polymerase (in grey). C. Strand displacement by φ29 DNA polymerase. D. ‘Second round’ of random hexamer primers annealing (in red) and extension by φ29 DNA polymerase (in blue).

### Polyomaviruses

**Historical background**
Polyomaviruses (PyVs) have been recognized as a separate virus family (Polyomaviridae) since 1999. Before that time they formed the genus *Polyomavirus* in the family *Papovaviridae* that contained the papillomaviruses (PA), polyomaviruses (PO) and the simian vacuolating agent (VA) 40 (SV40). Nowadays the latter virus forms the namesake species that is designated the polyomavirus type species (prototype) as listed in the ICTV 9th Report. The first members of the polyomavirus family are murine polyomavirus (MPyV) and SV40, identified halfway through the last century as filterable agents causing tumors in new-born mice and hamsters. The first two human polyomaviruses (HPyVs) were discovered in 1971. The JC-polyomavirus (JCPyV) was identified in a brain tissue extract from a progressive multifocal leukoencephalopathy (PML) patient with the initials J.C. The first BK-polyomavirus (BKPyV) was isolated from the urine of a nephropathic kidney
transplant patient with the initials B.K.. So far no compelling evidence was found for both JCPyV and BKPyV to be oncogenic in humans, despite being phylogenetically close to SV40 that can cause tumors in animals. Subsequently, more PyVs were identified in rodents, cattle and birds, but not in humans, until 2007, after which at least eleven new HPyVs have been identified (Table 1).

**TABLE 1. HUMAN POLYOMAVIRUSES**

<table>
<thead>
<tr>
<th>Name</th>
<th>Year identified</th>
<th>Source of isolation</th>
<th>Associated disease</th>
<th>Seroprevalence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>JC polyomavirus</td>
<td>1971</td>
<td>Urine, brain</td>
<td>Progressive multifocal leukoencephalopathy</td>
<td>40-80%</td>
</tr>
<tr>
<td>BK polyomavirus</td>
<td>1971</td>
<td>Urine</td>
<td>Nephropathy</td>
<td>80-100%</td>
</tr>
<tr>
<td>Washington University polyomavirus</td>
<td>2007</td>
<td>Respiratory tract</td>
<td>Not defined</td>
<td>40-95%</td>
</tr>
<tr>
<td>Karolinska Institute polyomavirus</td>
<td>2007</td>
<td>Respiratory tract</td>
<td>Not defined</td>
<td>60-90%</td>
</tr>
<tr>
<td>Merkel cell polyomavirus</td>
<td>2008</td>
<td>Skin lesion</td>
<td>Merkel cell carcinoma</td>
<td>40-80%</td>
</tr>
<tr>
<td>Human polyomavirus 6</td>
<td>2010</td>
<td>Skin</td>
<td>Not defined</td>
<td>70-85%</td>
</tr>
<tr>
<td>Human polyomavirus 7</td>
<td>2010</td>
<td>Skin</td>
<td>Pruritic rash, Thymic epithelial tumors</td>
<td>35-65%</td>
</tr>
<tr>
<td>Trichodysplasia spinulosa-associated polyomavirus</td>
<td>2010</td>
<td>Skin lesion</td>
<td>Trichodysplasia spinulosa</td>
<td>70-85%</td>
</tr>
<tr>
<td>Human polyomavirus 9</td>
<td>2011</td>
<td>Skin, blood, urine</td>
<td>Not defined</td>
<td>20-50%</td>
</tr>
<tr>
<td>Malawi polyomavirus , Human polyomavirus 10, Mexican polyomavirus</td>
<td>2012</td>
<td>Stool and skin lesion</td>
<td>Not defined</td>
<td>40-99%</td>
</tr>
<tr>
<td>St Louis polyomavirus</td>
<td>2012</td>
<td>Stool</td>
<td>Not defined</td>
<td>70%</td>
</tr>
<tr>
<td>Human polyomavirus 12</td>
<td>2013</td>
<td>Liver</td>
<td>Not defined</td>
<td>20%</td>
</tr>
<tr>
<td>New Jersey polyomavirus</td>
<td>2014</td>
<td>Muscle</td>
<td>Retinal blindness, Vasculitic myopathy</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

*In immunocompetents

**Phylogeny**

In 2011 the Polyomaviridae Study Group of the ICTV recommended to divide the single genus Polyomavirus into three genera, Orthopolyomavirus, Wukipolyomavirus and Avipolyomavirus, with the first two genera containing mammalian species and the latter only avian species. The committee in addition proposed a demarcation criterion for new polyomavirus species, with a whole genome sequence identity defined to be less than
81% compared to members of known species. Since this proposition dozens new PyVs have been identified. To accommodate these new viruses that represent putative new PyV species, in 2013 our group assembled and published a new, tentative polyomavirus phylogenetic tree based on the alignment of concatenated VP1, VP2 and LT amino acid sequences. An updated version thereof is shown in **FIGURE 3**.

**FIGURE 3. PHYLOGENETIC TREE OF POLYOMAVIRUSES.**
The unrooted phylogenetic tree consists of all (putative) polyomavirus species known until June 2014 and is based on the alignment of concatenated VP1, VP2 and LT amino acid sequences. The obtained branching pattern (topology) of basal nodes in the tree matches that proposed by Johne and colleagues (Johne et al., 2011). One distinct clade designated the **Avipolyomaviruses** contains only the bird PyV types. The other four clades, **Orthopolyomavirus-I** and -II, **Wukipolyomavirus** and **Malawipolyomavirus** consists of mammalian species. HPyVs are shown in red. Bar indicates number of substitutions per site. Numbers at branching events represent probability support values ranging from 0 (no support) to 1 (best support). Only probability support values lower than 1 are shown.

In addition to the 2011 tree, our new tree includes several new animal and human polyomaviruses, among which the HPyV9, MWPyV (including MXPyV and HPyV10), STLPyV, HPyV12 and NJPyV. Next to the Wukipolyomavirus and Avipolyomavirus genera proposed by Johne and coworkers, this new tree proposes to subdivide the **Orthopolyomavirus** genus in two lineages, **Orthopolyomavirus-I** and **II**, and add a fifth lineage called **Malawi-**
polyomavirus comprising MWPyV, MXPyV, HPyV10 and STLPyV. From **FIGURE 3** it is clear that the HPyVs (shown in red) do not form a monophyletic cluster. Rather they are distributed unevenly among four out of the five lineages. Whether the distribution of HPyVs among different genera should be considered the result of cross-species jumps of zoonotic viruses instead of virus-host coevolution, which was the most popular model of evolution for these viruses until few years ago when new HPyVs started to come to light, is unknown.

**Virion structure and genome organisation**

PyVs are non-enveloped icosahedral-shaped viruses with a capsid of 40-45 nm in diameter (**FIGURE 4A**). Five VP1 molecules interact with either a VP2 or VP3 molecule to form a capsomer. Subsequently, 72 capsomers auto-assemble into a capsid. Only VP1 is exposed to the surface of the capsid. The capsids are relatively resistant to formalin and heat inactivation and, since they are non-enveloped, also resistant to lipid solvents. The PyV capsid harbors a small double-stranded circular DNA genome wrapped with cellular histones. In the replicative phase the cell nucleus is packed with PyV capsids in a honeycomb-like structure (**FIGURE 4B**). The double-stranded circular PyV DNA genome of approximately 5,000 base pairs is one of the smallest among viruses and is divided into four regions, the non-coding control region (NCCR), the intergenic region, and the early and late coding regions (**FIGURE 4C**).

**FIGURE 4. POLYOMAVIRUS CAPSID AND GENOME.**

A. Model of the three-dimensional surface morphology of a polyomavirus capsid (adapted from Salunke et al., 1986). B. TSPyV capsids present in the nucleus of an inner root sheath cell. C. Schematic representation of the polyomavirus genome.

The NCCR contains the origin of replication (ori) flanked by several large T-antigen-binding sites (GAGGC sequence repeats), as well as the transcription promoters and regulatory elements. In individuals with disseminated JCPyV or BKPyV-infection, the viral genome detectable in different body compartments is known to vary. NCCRs of isolates obtained...
from blood or cerebrospinal fluid can vary from what is called the ‘archetype’ NCCR that is found in urine and thought to belong to transmissible virus. It is believed that these NCCR rearrangements increase T-antigen transcription and the virus replication rate, as was shown for instance for JCPyV.\textsuperscript{86} Systematic analyses of the NCCR region among isolates obtained from different body compartments within individuals have not been reported yet for the new HPyVs.

HPyV DNA replication takes place in the cell nucleus. The early genes, the T-antigens, are expressed prior to the onset of DNA replication. The late region-encoded genes found on the opposite strand, agno and VP1, VP2 and VP3, are expressed after DNA replication has begun and are regulated by the T-antigens. At the 3’ end, the early and late regions are separated by a small intergenic region (\textit{FIGURE 4C}).

The genome numbering system for PyVs differs among species, with a SV40-like numbering system having nucleotide position 1 located in close proximity of the second large T binding site upstream of the AT-rich region (TATA box), or with a HPyV6-like numbering system with nucleotide position 1 preceding the nucleotide A of the T-antigen start codon. For all HPyVs, except for MCPyV, the numbering proceeds in direction of the late region. For MCPyV, like MPyV, the genome is numbered the other way around.

\textbf{Transcription of early and late genes and microRNA expression}

Polyomavirus early and late mRNAs are transcribed through alternative splicing by the host cellular splicing machinery and are terminated by posttranscriptional processing at the polyadenylic acid (poly(A)) tail site generally located in the intergenic region. By alternative splicing of primary mRNAs which creates frame shifts, the small PyV genome with overlapping open reading frames (ORFs) is optimally exploited. Each PyV generates at least two early mRNAs encoding for the large and small T-antigens (LT and ST), as illustrated in \textit{FIGURE 5}. In rodents, an additional early splice product is found encoding for middle T-antigen (MT). For many years it was believed that the ST, MT and LT splice products were the prime early mRNA products. Some additional early mRNAs were identified revealing a secondary splice event in the LT-mRNA, which causes truncations of the helicase domain of LT, for example 17kT, 57kT and TruncT (\textit{FIGURE 5}).\textsuperscript{87-90} The exact role of these additional transcripts remains unknown, although for the SV40 17kT product stimulation of cell proliferation was shown.\textsuperscript{90,91} For MPyV a fourth early splice product was identified using the LT splice donor site combined with the MT acceptor site, called tiny T.\textsuperscript{92} Coinciding with the research described in this thesis, for MCPyV an alternative T-antigen was identified named ALTO,\textsuperscript{93} which is encoded in the +1 frame of the second exon of LT. This phenomenon, known as overprinting, allows encoding of two separate protein sequences and is commonly seen in viruses.\textsuperscript{94-97} Translation of ALTO is established by using an alternative internal start-codon, possibly guided by an internal ribosome entry site (IRES). For most of the other new HPyVs the transcription-patterns have not been reported yet.
The late mRNA transcript is transcribed from the other strand in opposite direction. The late genes encode for the capsid proteins VP1, VP2 and VP3, and encode agnoprotein in JCPyV and BKPyV and in several animal PyVs but not in less unrelated (non-Orthopolyomavirus-II genus) HPyVs. The precise role of agnoprotein is not known yet, although in vitro interaction between agnoprotein and proliferating cell nuclear antigen (PCNA) has been described, which interferes with the PCNA-dependent DNA synthesis. It has been postulated that PCNA-bound agnoprotein might have an inhibitory effect on viral DNA replication late in infection, which might promote capsid assembly. The late mRNA transcript uses alternative internal start codons for encoding of the structural VP1,
VP2 and VP3 proteins, the latter protein VP3 is translated in the same ORF as VP2. Strong evidence for the presence of two internal ribosome entry sites (IRES) located between the VP2 and VP3 start codons is described for SV40.99 The VP3 protein is not present in all PyVs. Lack of VP3 expression is described for MCPyV and sequence analysis suggests that two sub-clades of PyVs within the Orthopolyomavirus-I genus, including MCPyV and NJPyV, lack VP3 expression.100 For TSPyV the VP3 protein is detected in silico.

The late mRNA transcripts of SV40, MPyV, JCPyV, BKPyV and MCPyV also encode for microRNA (miRNA), which is antisense to the early transcripts.101-103 Read-through beyond weak late strand poly A signals most likely generates miRNA, although recently evidence of an independent miRNA promoter is given for MCPyV located within early strand coding sequences.104 Whether the new HPyVs, next to MCPyV, also possess miRNAs is not known yet, but for TSPyV a high-scoring pre-miRNA structure was predicted.105 miRNAs do possess the ability to cleave early transcripts via a siRNA-like mechanism. The PyV miRNA may function as a negative feedback loop tapering T-antigen expression in later phases of infection. PyV miRNAs probably also target cellular genes involved in immune responses, such as the stress-induced ligand ULBP3 and thereby influencing (antiviral) NK cell activity.106 In Merkel cell carcinomas, MCPyV-encoded miRNAs were identified that potentially regulate T and B cell receptor signaling hampering viral (tumor) immune recognition.107

### T-antigens: structure, function and involvement in transformation

The T-antigens play essential roles in viral transcription and replication, as well as in host cell tuning to enable virus replication.108,109 As polyomaviruses are completely dependent on the host cell replication machinery, a significant part of this tuning is aimed at inducing S-phase and bypassing cell cycle-control measures. In some cases, these features can result in uncontrolled cell growth and even tumor formation. So far, MCPyV is the only new HPyV for which these (potentially) oncogenic phenomena have been noted,110 although very recently HPyV7 was detected in thymic epithelial tumors.64 The T-antigens of MCPyV have been studied already quite extensively, whereas for the other new HPyVs this knowledge is almost entirely based on in silico analyses.
FIGURE 6. FUNCTIONAL DOMAINS AND BINDING PARTNERS OF SV40 SMALL T, MPYV MIDDLE T AND SV40 LARGE T.

The HSC70 protein binds to the J-domain of ST, MT and LT and PP2A binds via zinc-binding domains (Zn) to ST and MT. The interaction of SRC kinases with PP2A in MT results in phosphorylation of tyrosines (Y) and serine (S) which set off MT binding to signal transduction pathway products (e.g., SHC, PI3K, 14-3-3 and PLCγ1). The proline-rich region in MT is depicted as PPP and φ represents the transmembrane domain. Several cellular proteins like BUB1, IRS1 and CUL7 interact with LT. The tumor suppressor genes pRB and p53 bind to the LXCXE motif and to the helicase domain of LT, respectively. Karyopherin (KPNA) which plays a central role in transporting molecules between the cytoplasm and the nucleus, binds to the nuclear localization signal (NLS). The DNA binding domain (DBD) and helicase domain important for replication initiation, recruit cellular replication factors such as DNA polymerase-α catalytic subunit (POLA), the DNA primase complex (PRIM), the replication protein A complex (RPA) and DNA topoisomerase 1 (TOP1). The LT C-terminal phosphorylated threonine residue binds to the ubiquitin ligase Fbw7. (Adapted from Cheng et al., 2009)115

In FIGURE 6 a schematic overview is given of the T-antigens with their functional domains and binding partners (cellular factors), as described for SV40 (ST and LT) and MPyV (MT). ST, MT and LT share their N-terminus (first exon of LT) while having C-terminal regions of different sizes encoded in different reading frames that are expressed by alternative splicing (FIGURE 5). The new human polyomaviruses seem no exception to this rule. This shared region occupied by the so-called J-domain contains important motifs for virus replication and cellular transformation, such as DnaJ.109 Downstream, ST contains a PP2A subunit-binding motif. ST-PP2A binding most likely activates the Akt-mTOR pathway resulting in (tumor) cell survival.111,112 LT further includes a number of functionally important sequence signatures: a nuclear-localization signal, a DNA-binding domain (DBD) and an ATPase domain-containing helicase domain.109,113 Studies of SV40 LT show
that the DBD of LT directly bind to ‘GAGGC’ sequence repeats present in the ori of the virus
where it forms hexamers and functions as a helicase to unwind the viral DNA and enable
replication.\textsuperscript{114} SV40 LT moreover has a pRB-binding motif as well as a p53-interaction
domain that play a role in cellular transformation.\textsuperscript{115}

In rodents, the MT protein is recognized as the most transforming T-antigen. MPyV MT is
known to cause multiple tumors in new born mice, and mammary, skin and bone cancer
in adult mice.\textsuperscript{116} MT shares the N-terminal J-domain with ST and LT, and the PP2A-binding
domain with ST. After splicing MT has a unique C-terminal part which is encoded by a \textit{de
novo} ORF that overlaps with the second exon of LT encoded in an alternative reading frame.
This unique, C-terminal part of MT is reminiscent to ALTO (\textit{FIGURE 5}).\textsuperscript{93} The MT protein is
phosphorylated by Src tyrosine kinase interacting with PP2A-bound MT. Phosphorylated
MT induces binding and activation of members of the signal transduction pathway like
the Shc1 phosphotyrosine docking protein, the 14-3-3 protein, phospholipase C (PLC\textgamma) and
phosphatidylinositol 3-kinase (PI3K).\textsuperscript{115-117}

The C-terminal part of MT is proline-rich, contains a transmembrane domain at the very
C-terminal end which is important for transformation, and is intrinsically disordered.
Intrinsically disordered proteins lack a tertiary structure and are known as major regulators
of diverse biological functions and are often related to pathogenesis, including cancer.\textsuperscript{118}
Sequence analysis of MCPyV ALTO also revealed these characteristics, but to what extend
ALTO has transforming activities is not known yet. The \textit{de novo} ORF encoding for ALTO
and the second exon of MT is restricted to a substantial part of the Orthopolyomavirus-I
genus.\textsuperscript{119} Interestingly, all naturally oncogenic polyomaviruses noted so far, e.g. MCPyV,
MPyV, Hamster polyomavirus (HaPyV) and Raccoon polyomavirus (RacPyV), belong to this
clade.

\textbf{Polyomaviruses and disease association}

In general, HPyVs are ubiquitous viruses that infect their host without causing clinical
symptoms. Primary infection is followed by a persistent, asymptomatic (latent) infection
with low levels of replication. In case of impaired immune function, for example as a result
of AIDS or the use of immunosuppressive drugs, HPyV infection can become symptomatic
and cause severe disease.

So far, four HPyVs have been associated with disease. JCPyV causes PML, a rare life-
threatening demyelinating disease of the central nervous system observed primarily in bone
marrow transplant patients,\textsuperscript{46} AIDS patients,\textsuperscript{45} and natalizumab-treated multiple sclerosis
patients.\textsuperscript{47} Natalizumab-treated patients are not immunocompromised in general, but
due to the targeted effect on adhesion molecules in cells lining the intestines and brain,
focal cellular immunodeficiency is achieved.\textsuperscript{120,121} Polyomavirus-associated nephropathy
(PVAN) is an important cause of renal allograft dysfunction and graft loss, resulting from
high-rate lytic BKPyV replication in the transplanted kidney.\textsuperscript{53,54} Merkel cell polyomavirus
(MCPyV) identified from the Merkel cell carcinomas (MCCs) in 2008,\(^{59}\) is one of the novel HPyVs associated with disease. Current data strongly suggest that the majority of MCCs harbor clonally integrated and expressed viral genome copies that contribute to MCC carcinogenesis.\(^{59,88,122,123}\) TSPyV, identified in 2010 and subject of this thesis is associated with the skin disease TS introduced earlier. For the other new HPyVs pathogenicity is still unknown, although for Human polyomavirus 6 (HPyV6), 7 (HPyV7) and for New Jersey polyomavirus (NJPyV) indications for disease association have recently been found. HPyV6 is detected in BRAF inhibitor-induced epithelial proliferations including cutaneous squamous cell carcinomas,\(^{124}\) HPyV7 in pruritic rash and thymic epithelial tumors,\(^{63,64}\) and NJPyV in a patient with vasculitic myopathy.\(^{80}\) Karolinska Institute polyomavirus (KIPyV) and Washington University polyomavirus (WUPyV) are regularly detected in respiratory samples,\(^{125}\) but no clear association with respiratory disease is found at the moment.\(^{126}\) We recently described the presence of Human polyomavirus 9 (HPyV9) DNA in kidney transplant patients.\(^{127}\) Whether HPyV9 is pathogenic in immunocompromised patients, alone or in concert with the well-known nephropathogen BKPyV deserves further study. Whether the (putative) pathogenicity of the new HPyVs is caused by reactivation, like observed for JCPyV and BKPyV, or by a primary infection is currently not known.

**Seroprevalence of polyomavirus infection**

In general upon a viral infection, humoral immune responses are induced resulting in the production of specific IgM and IgG immunoglobulin antibodies detectable in serum. Detection of specific serum antibodies serves as an important marker of infection and has become instrumental in studying the progression of a certain infection. A classical antiviral antibody response starts with IgM production immediately followed by IgG production. T-cell help is needed to accomplish the Ig-class switch from IgM to IgG. The IgM response is present for a short period, usually some 3 months, while the IgG response usually persists and may be boosted during a secondary infection or reactivation of the virus. Over time the avidity of IgG antibodies for their antigen will increase, whereas IgM is not detected anymore.

PyV sero-epidemiological studies in humans are mainly based on IgG seroresponses directed against VP1, the immunodominant major capsid protein.\(^{51}\) Two methods are commonly used to express VP1 antigen; one is based on generation of glutathione-S-transferase (GST)-HPyV VP1 fusion proteins,\(^{128}\) the other on VP1 virus-like particles (VLP). Both antigen preparations can be bound to Luminex beads or ELISA plates.\(^{50,61,62,129}\) Although differences in the expression of conformational epitopes was anticipated between these methods, HPyV seroprevalences measured by both methods are remarkably similar.\(^{66,67}\) Cross-sectional serological studies of the well-known JCPyV and BKPyV have shown that both infections are highly prevalent in the general population and primary
infection generally occurs in (early) childhood. In TABLE 1 an overview is given of the seroprevalences found in immunocompetent adults for JCPyV, BKPyV and for the new HPyVs. Seroprevalences of HPyV differ among species, with BKPyV reaching almost 100% and HPyV12 limited to 20%. Geographical differences with respect to HPyV species-specific seroprevalences, for instance among continents, seem small. To what extent HPyV seroprevalence and intensity of seroresponses is related to tissue tropism, target organ or frequency of reactivation is not known.

Polyomavirus persistence, reactivation, and spread of infection
In healthy individuals, the detection rate of HPyV DNA is generally much lower than their seroprevalence. In urine of healthy blood donors for example, the detection rate of JCPyV and BKPyV DNA is approximately 3 and 10-fold lower than the seroprevalence measured within the same group, respectively. In immunosuppressed solid organ transplant patients and hematopoietic stem cell recipients, however, BKPyV can be found in urine in the majority of (seropositive) cases, often in very high amounts indicative of massive reactivation of infection. For the new HPyVs the association between impaired immunity and reactivation is less clear. For the occurrence of MCPyV-positive Merkel cell carcinomas a weaker association with immunosuppression is found. Also elderly are at increased risk of developing this tumor, which is possibly explained by immunosenescence. The MCPyV DNA detection rates on the skin do not differ much between immunocompetents and immunocompromised, and resemble the seroprevalence of 50%. In general, it should be noted that MCC is a very rare condition implying that (host) factors other than immunity probably also play a role in controlling infection and transformation by this virus. For the other new HPyVs the relation of manifest disease with reduced immunity is unclear at the moment. This is largely explained by the absence of an established clinical condition attributed to one of these new viruses.

Which route of virus excretion primarily drives HPyV infection is not precisely known, but the efficiency of infection seems clear from the high seroprevalence numbers, both for the known and the new HPyVs. From studies of JCPyV and BKPyV it is known that polyomaviruses are found in urine, feces and saliva. The load of excreted virus is remarkably high, such that these HPyVs even serve as markers of sewage pollution of for instance recreational waters. One of the new HPyVs, MCPyV, has been frequently and abundantly detected in urban wastewater. Regarding possible routes of excretion and transmission of cutaneous MCPyV, a recent study showed the presence of viral DNA on 75% of samples from environmental surfaces (door handles, ticket machines etc). DNase sample treatment and viral load measurement suggested that about 5% of the detected MCPyV DNA was protected, probably encapsidated and therefore potentially infectious.
Outline of this thesis

As described in the preceding Introduction, several new human polyomaviruses have recently been identified. This thesis describes the discovery of the trichodysplasia spinulosa-associated polyomavirus (TSV or TSPyV) and its (sero)prevalence, and characterizes several aspects of its infection. Furthermore, it describes the TSPyV T-antigen transcriptome and proteome, and reveals the putative role of TSPyV LT in inner root sheath cell hyperproliferation.

Chapter 2 describes the basis of this thesis, the discovery of a new human polyomavirus called TSPyV in an immunocompromised heart transplant patient with TS. Its genome and its main antigens are outlined. Furthermore, the clinical course and response to treatment is described, and the prevalence of TSPyV in immunocompromised patients.

Chapter 3 describes the development and validation of a Luminex-based TSPyV VP1 immunoassay. With this method the seroprevalence of TSPyV within immunocompetent and immunosuppressed populations was determined.

Chapter 4 reports the differences in serological profile between TSPyV and four other recently identified HPyVs found on the skin, being MCPyV, HPyV6, HPyV7 and HPyV9.

Chapter 5 describes a new Dutch case of TS. In this case it is shown that TS is caused by a primary disseminated TSPyV infection with viral DNA detectable in various body compartments which precedes seroconversion.

Chapter 6 thoroughly characterizes the TSPyV T-antigen transcriptome and proteome, in human cell lines as well as in lesional TS tissue, and underscores the unique position of TSPyV among other HPyVs indicated by for instance MT and ALTO expression.

Chapter 7 investigates the hyperproliferative nature of TS lesions and addresses the relationship between TSPyV LT and cellular cell-cycle regulators such as pRB, p16 and p21.

Chapter 8 summarizes and discusses the general findings of the research described in this thesis in relation to TSPyV-induced infection and disease.