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# Antibodies reacting with JCPyV\_VP2\_167-15mer as a novel serological marker for JC polyomavirus infection

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## Abstract

**Background:** JC polyomavirus (JCPyV) is a widespread human polyomavirus that usually resides latently in its host, but can be reactivated under immune-compromised conditions potentially causing Progressive Multifocal Leukoencephalopathy (PML). Detection of antibodies against the major capsid protein VP1 currently is the main marker for assessment of infection with JCPyV.

**Methods:** Based on a peptide microarray, peptide JCPyV\_VP2\_167-15mer was selected and a peptide ELISA was developed for detection of antibodies directed against this peptide. Epitope mapping and computational modelling was performed to further characterize this peptide. In a cohort of 204 healthy subjects it was investigated whether antibodies against JCPyV\_VP2\_167-15mer were correlated with VP1 serology or urinary viral load.

**Results:** Epitope mapping of peptide JCPyV\_VP2\_167-15mer showed that the minimal epitope consisted of L<sub>173</sub>PALTSQEI<sub>181</sub> with amino acids P<sub>174</sub>, L<sub>176</sub> and E<sub>180</sub> being essential for antibody recognition. Computational analysis was used to predict that this epitope is located at an exposed domain of the VP2 capsid protein, readily accessible for immune recognition upon infection. No correlation could be observed with JCPyV VP1 antibody levels, or urinary viral load.

**Conclusion:** This work indicates that specific antibodies against JCPyV\_VP2\_167-15mer might be considered as a novel serological marker for infection with JCPyV.

**Keywords:** JC Polyomavirus, Biomarker, Peptide serology, VP2, Progressive Multifocal Leukoencephalopathy

## Introduction

JC Polyomavirus (JCPyV) is a human neurotropic polyomavirus that was found to be the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease [1-4]. JCPyV can switch from its latent state to an activated state in immunocompromised subjects such as HIV-1 infected patients and in multiple sclerosis (MS) patients treated with natalizumab [5-7]. The JC Polyomavirus capsid is composed of 72 pentamers of the major capsid protein VP1, with one of the minor coat proteins (VP2 or VP3) in the center of each pentamer. Both minor proteins are essential for the viral life cycle [8,9] and were shown to act as membrane

proteins during infection and to form pores in host cell membranes [10].

Antibodies to JCPyV VP1 are widely prevalent in healthy subjects indicating that most individuals have been exposed to or are latently infected with the virus [11-17]. Antigenic epitopes have been described for VP1, with most of these epitopes being shared between JCPyV and the other polyomaviruses BKPyV and SV40 [18]. Despite this epitope sharing, JCPyV specific serology assays using full length recombinant JCPyV VP1 as antigen were developed. Specificity was shown by inhibition experiments using VP1 from other known polyomaviruses [19-21]. Serological results should however be interpreted with caution as serological cross-reaction with closely related, yet unidentified human polyomaviruses can never be excluded [22-24]. Currently, the STRATIFY JCPyV ELISA using baculovirus-expressed VP1

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virus-like-particles (VLP) as antigen, is the only Food and Drug Administration (FDA) approved assay for JCPyV [12,25].

Little attention has been paid so far to JCPyV VP2 or VP3 as immunogenic proteins, although some examples have been described of the immunogenic nature of these minor capsid proteins in other polyomaviruses. A high prevalence of antibodies against VP2 has been described for WU Polyomavirus [26]. Furthermore, a linear epitope was identified in SV40 VP2/VP3 that showed immunoreactivity in serum from 21.9% of blood donors [27]. Also BKPyV VP2 and VP3 were identified as targets of cellular immunity [28]. Peptide microarray analysis using a comprehensive set of polyomavirus derived peptides demonstrated that several non-VP1 peptides were recognized by antibodies in human plasma and could potentially represent linear epitopes of these proteins [29].

In this work we have investigated, using a peptide microarray setup, whether linear epitopes could be identified in JCPyV VP2. A 15-mer peptide was identified that was thereafter used for the development of a peptide ELISA. The immunoreactivity of this peptide was further characterized and its relationship with other JCPyV markers was investigated.

## Results and discussion

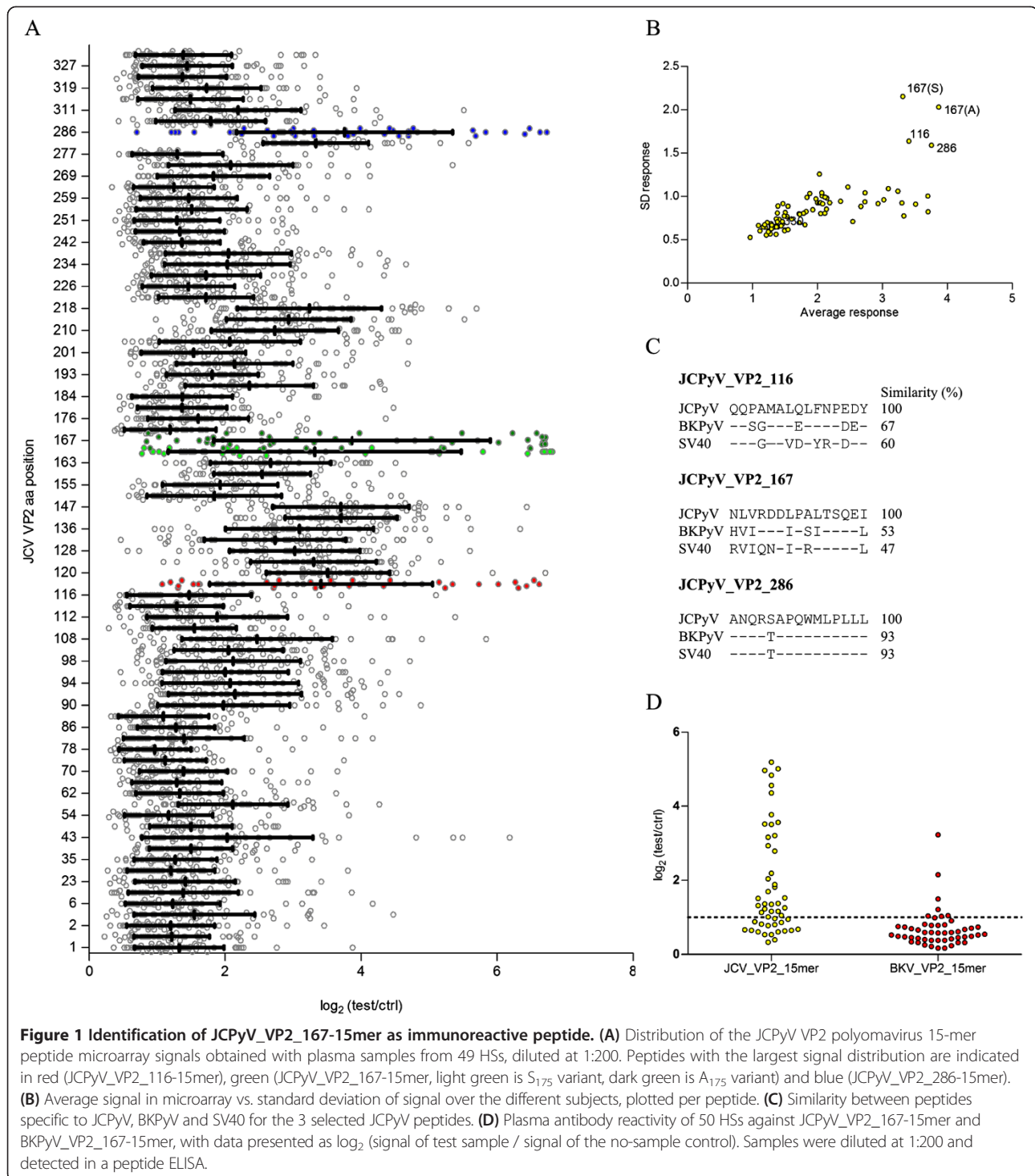
A total of 82 15-mer peptides derived from JCPyV VP2 were incubated in a peptide microarray format with plasma samples from 49 healthy subjects (HS), resulting in 4018 data points. All individual data points were plotted per peptide and the mean value and standard deviation was calculated per peptide (Figure 1A). Upon plotting these descriptive statistics on an x-y plot, 4 peptides clearly had different responses compared to the other peptides, with high average response and high variation over the different subjects (Figure 1B). These peptides were JCPyV\_VP2\_116-15mer, JCPyV\_VP2\_167-15mer (variant with S<sub>175</sub> and variant with A<sub>175</sub>) and JCPyV\_VP2\_286-15mer. Remarkably, two variants of the same peptide (JCPyV\_VP2\_167-15mer) both had similar results, suggesting that the variant position is not involved in the epitope recognition. Since JCPyV VP2 is highly homologous to BKPyV VP2 and SV40 VP2, sequence similarity was assessed between the identified JCPyV peptides and the corresponding peptides in BKPyV and SV40 (Figure 1C). For peptide JCPyV\_VP2\_167-15mer this analysis showed that the corresponding SV40 peptide overlaps largely with a peptide that was identified earlier as an epitope that is recognized by antibodies in serum samples from healthy donors carrying SV40 [27]. As there is also low sequence homology of this peptide with the corresponding BKPyV and SV40 peptides, we decided to further investigate this specific JCPyV peptide. In order to verify that human plasma samples contain IgG

antibodies that react with this specific peptide, a peptide ELISA was set up using the synthetic peptides JCPyV\_VP2\_167-15mer and BKPyV\_VP2\_167-15mer. This ELISA was used to test for anti-peptide antibody responses in plasma from 50 HSs that had been diluted 200-fold (Figure 1D). This confirmed that plasma from healthy individuals indeed contains IgG antibodies reacting with this peptide, with an overall prevalence of 64% (32/50) for JCPyV\_VP2\_167-15mer and only 14% (7/50) for BKPyV\_VP2\_167-15mer.

Molecular modelling was performed on a 36-mer, covering amino acids 154 to 189 of JCPyV VP2 (Figure 2). This modelling predicts that this peptide is characterized by a stable secondary structure consisting of 2  $\alpha$  helix domains flanking a smaller exposed amino acid stretch. This computational modelling indicates that the majority of JCPyV\_VP2\_167 is located in an exposed region of the VP2 protein and as such this physical configuration might favour its immunological reactivity, in line with the results observed in the peptide ELISA.

In order to better characterize the immunoreactivity of this JCPyV VP2 derived peptide, epitope mapping experiments were performed. For this investigation, a peptide ELISA was employed to test 3 plasma samples that were highly reactive towards JCPyV\_VP2\_167-15mer using different specifically designed synthetic peptides. On the one hand a series of 7-mers, 9-mers, 11-mers and 13-mers, overlapping with all but one residue was tested (Figure 3A). This test showed that a minimal peptide required to retain immunoreactivity consisted of the sequence LPALTSQEL. This observation was confirmed by the analysis of anti-peptide antibody responses in plasma from 50 HSs using the 9-mer, 11-mer, 13-mer and 15-mer that was found to retain immunoreactivity (Figure 3B). On the other hand each of the amino acids in JCPyV\_VP2\_167-15mer was replaced individually by alanine (alanine walking) (Figure 3C). Using this approach the contribution of each specific residue used to the immunoreactivity of the peptide is determined [30,31]. The data obtained using these peptides in the peptide ELISA indicate that three residues in JCPyV\_VP2\_167-15mer are important for antibody recognition: P<sub>174</sub>, L<sub>176</sub> and E<sub>180</sub>. Replacement of any of these residues by alanine resulted in the complete elimination of immunoreactivity of the peptide. These data also confirmed the results of the previous experiment as all essential amino acid residues are located within the minimal peptide affirmed. Remark that the corresponding BKPyV peptide (IPSITSQEL) has an isoleucine residue at position 176 instead of leucine.

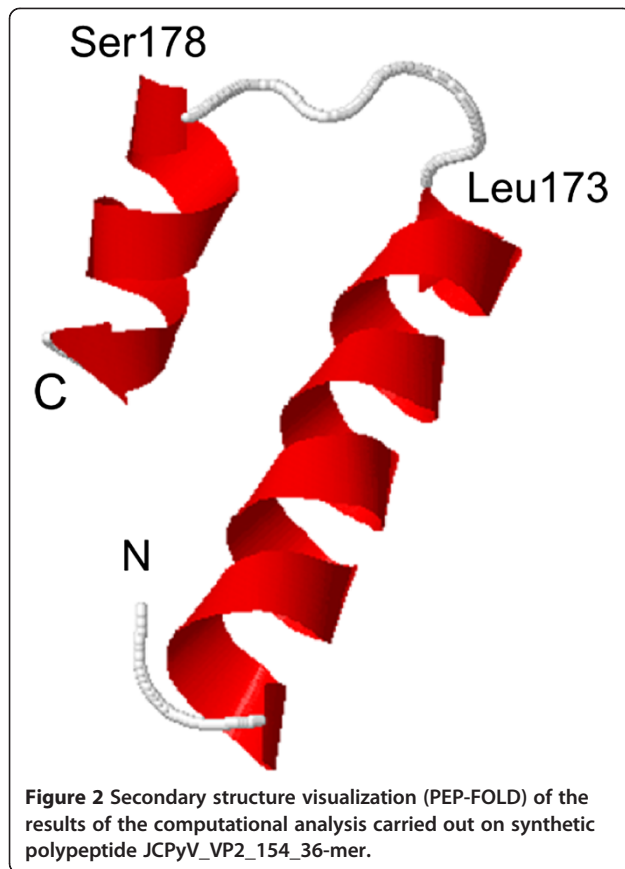
Since several mutations have been described in the VP2 region of JCPyV, it might be of interest to investigate whether some of these mutations are located in the JCPyV\_VP2\_167-15mer. A first mutation that is known, is the E<sub>172</sub> mutation [32]. However, this residue is not



located in the minimal epitope and as such is unlikely to play a role in antibody recognition. A second mutation is the S<sub>175</sub> mutation which was already part of our initial investigation on the peptide array. Based on both the array data and the epitope mapping data, it is clear that this residue also does not play a role in antibody recognition. Taken together, we might conclude that the

peptide ELISA using the JCPyV\_VP2\_167-15mer NLVRDDLPA<sup>L</sup>TSQEI is capable of detecting antibodies against all JCPyV strains, independent of the strain specific sequence of this VP2 region.

The data presented above are demonstrative of the immunoreactivity of JCPyV\_VP2\_167-15mer and its minimal epitope LPALTSQEI. This does however not imply that

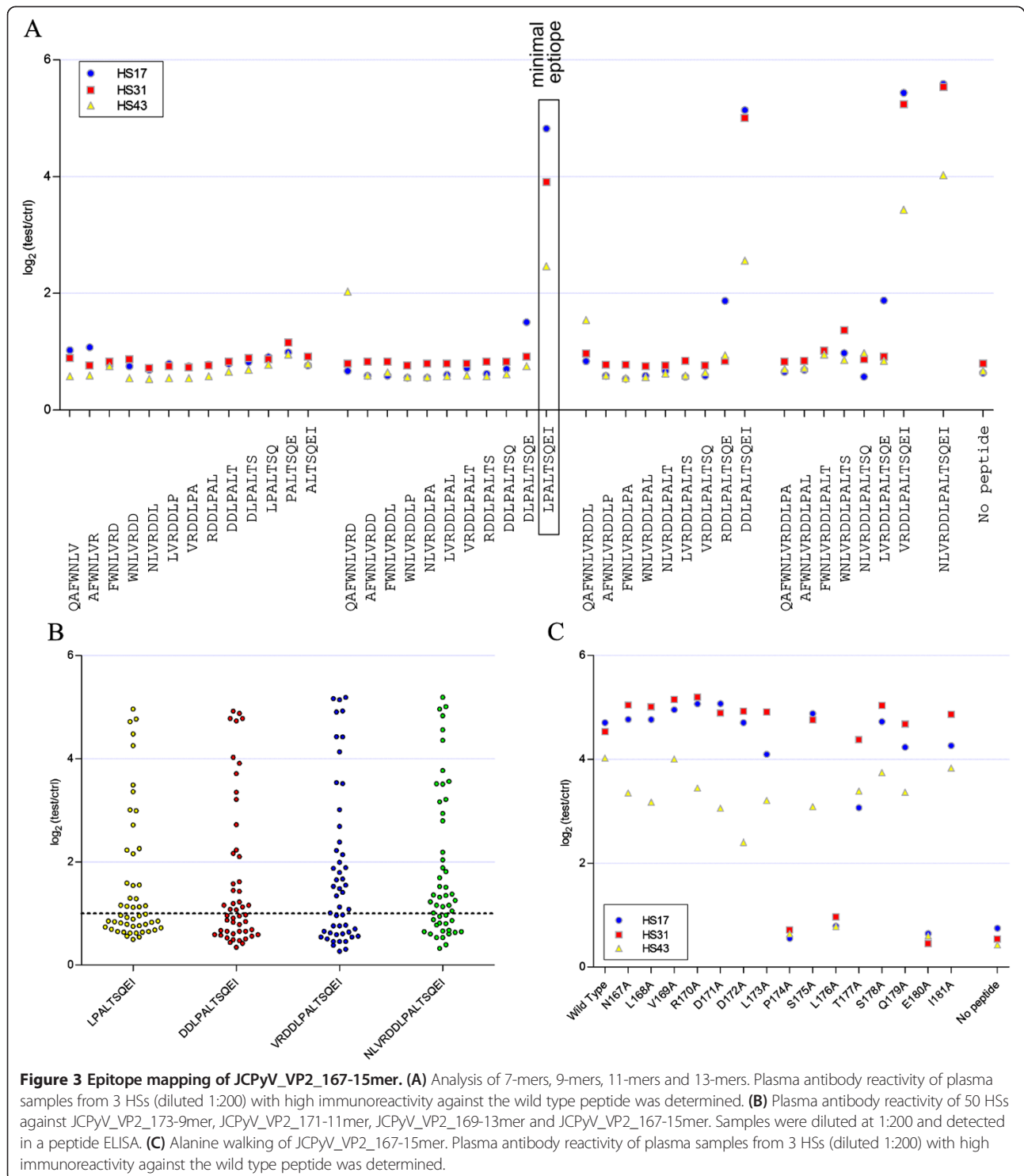


the immune responses observed are elicited by exposure to or infection with JCPyV. Any protein containing the LPALTSQEI sequence or closely related sequence might in fact be the antigen responsible for the positive immune responses observed. The responses observed with JCPyV\_VP2\_167-15mer would then be non-specific responses. In order to investigate the possibility that a protein from another organism might be at the basis of the observed responses, a protein BLAST search was performed against the non-redundant protein database using JCPyV\_VP2\_171-11mer as query sequence [33] (Additional file 1: Table S1). Within the Viridae only SA12 VP2 and BKPyV VP2 were identified, respectively with 1 or 4 mismatches compared to the query sequence. Also in the human proteome no proteins were identified with less than 4 mismatches. Most proteins with less than 4 mismatches compared to JCPyV\_VP2\_171-11mer, however, were found in Bacteria and Animalia. Also one protein from protozoan origin and one protein from a grass species were identified. Of all identified sequences the corresponding 15-mer peptide was synthesized and used for assessment of their immunoreactivity in 3 plasma samples that were highly reactive towards JCPyV\_VP2\_167-15mer. Out of the 30 peptides tested, 9 showed some response in the peptide

ELISA that were similar as JCPyV\_VP2\_167-15mer (Figure 4). The strongest signals were observed for isopropylmalate isomerase from *Micrococcus luteus*, anti-FecI sigma factor FecR from *Spirosoma linguale* DSM 74, hypothetical protein from *Beggiatoa alba*, putative peroxisomal biogenesis factor from *Ixodes scapularis* and probable E3 ubiquitin-protein ligase ARI1-like from *Brachypodium distachyon*. Although it cannot be excluded, it is very unlikely that one of these proteins is responsible for the immune response observed in the peptide ELISA using JCPyV\_VP2\_167-15mer. First of all some of these organisms are either rare or occur only at specific locations. Secondly, the proteins identified are not expected to be strong antigens as they are known to be located intracellularly and as such are unlikely to be exposed to the host's humoral immune system. All together, these data suggest that an immune response against JCPyV VP2 can be observed in HSs and that the amino acid stretch with sequence LPALTSQEI functions as an epitope on this protein.

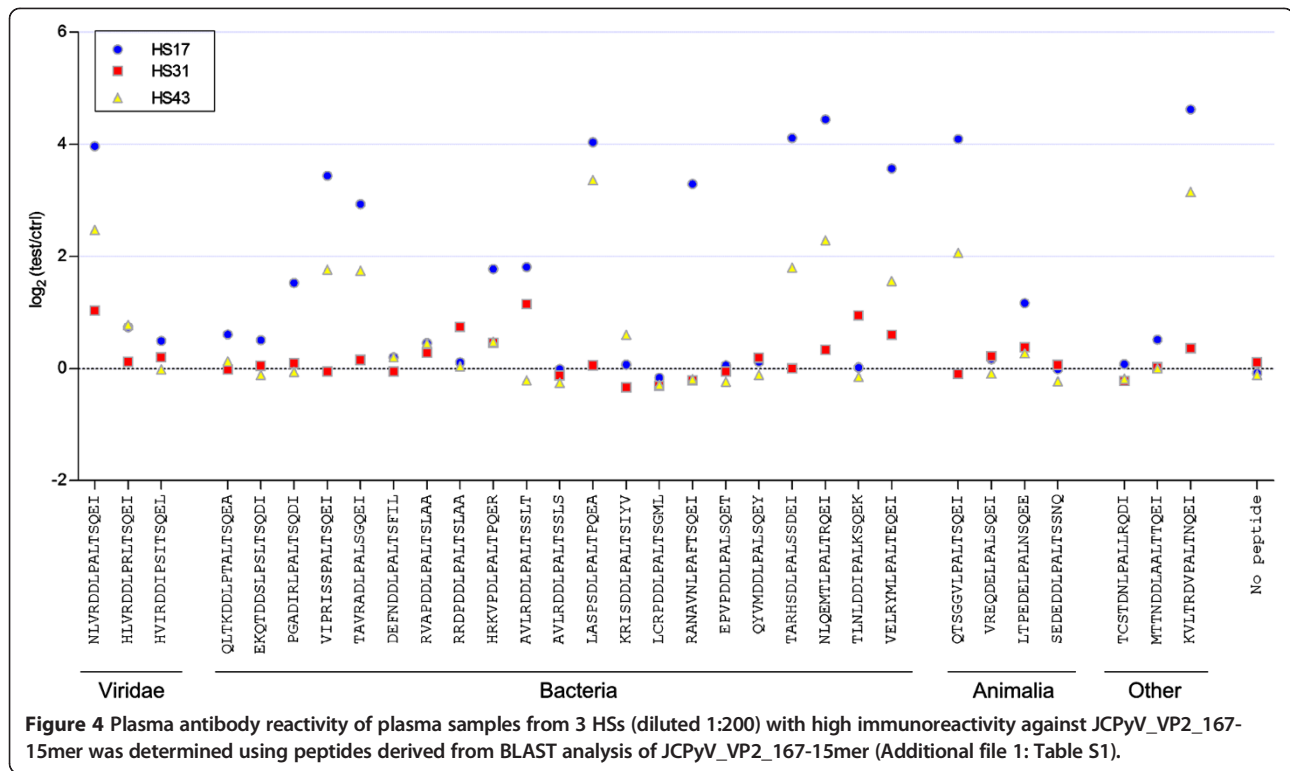
Based on these data, we believe JCPyV\_VP2\_167-15mer truly acts as an epitope and therefore the peptide ELISA using JCPyV\_VP2\_167-15mer as antigen was employed to analyse the immune response against this peptide in a larger cohort of 204 HSs (Figure 5). As was already seen in the smaller cohort of 50 HSs, there was a large variation over the different subjects. Subjects were further categorized based on their JCPyV VP1 serology and JCPyV urinary viral load (Figure 5A). However, no difference ( $P > 0.05$ ) between JCPyV VP1 seropositive and seronegative subjects could be observed. The same was observed when comparing subjects shedding virus in their urine and virus with a negative urinary viral load. Although we have shown before there is no cross-reactivity with BKPyV, we investigated the possibility that infection with BKPyV was interfering in the assay. Therefore, also BKPyV VP1 antibody levels were determined and subjects were categorized based on their JCPyV VP1 and BKPyV VP1 serology (Figure 5B). Again, no difference ( $P > 0.05$ ) was observed between any of the groups. It is known urinary viral load and JCPyV VP1 antibody levels are correlated [34]. We have investigated whether this is also true for JCPyV\_VP2\_167 antibody levels (Figure 5C). In contrast to VP1 antibody levels, no correlation was found between urinary viral load and JCPyV\_VP2\_167 antibodies ( $P > 0.05$ ). It was also apparent that JCPyV\_VP2\_167 antibody levels were not correlated with donor age ( $P > 0.05$ , Figure 5D). It will also be of interest for future studies to investigate whether JCPyV\_VP2\_167 antibody levels remain constant over time as is the case for VP1 antibody levels [35,36].

The lack of correlation between JCPyV\_VP2\_167 and JCPyV VP1 antibody levels is an interesting, but surprising



finding. It is however not the first time that JCPyV VP1 serology is debated for its use as the sole infection marker as several examples have been described where JCPyV DNA was detected in urine, plasma or specific blood cell populations of individuals who were

seronegative for JCPyV VP1 [37-40]. In recent work, also a lack of correlation was observed between the level of anti-JCPyV antibody and anti-JCPyV neutralizing activity as 28.6% of JCPyV VP1 Ab<sup>+</sup> samples were not capable of neutralizing the *in vitro* infection of



COS7 cells by JCPyV [41]. Further research would however be needed to investigate whether JCPyV\_VP2\_167 antibodies are capable of neutralizing JCPyV infection.

It might be debated whether JCPyV VP2 can be recognized by the immune system as it was proposed that VP2 is located at the inner side of the capsid [42]. Crystal structure analysis however showed that the N-terminal part of VP2, including the JCPyV\_VP2\_167-15mer region is not tightly folded and retains high flexibility, making it easier for VP2 to emerge from inside the virion [43,44]. The fact that it was also shown that 52.3% of serum samples show immunoreactivity with WU Polyomavirus VP2 and 21.9% of blood donors react with SV40 VP2 derived peptides, strengthens the conclusion that JCPyV VP2 acts as an antigen recognized by the immune system upon JCPyV infection [26,27]. We postulate that the peptide studied in this work, JCPyV\_VP2\_167-15mer, acts as one of the epitopes responsible for the antigenic properties of JCPyV VP2.

## Conclusions

The work presented here shows that plasma from a large portion of HSs reacts specifically with a peptide derived from JCPyV VP2 capsid protein. Epitope mapping experiments demonstrated that the minimal epitope consisted of L<sub>173</sub>PAALTSQEI<sub>181</sub> with amino acids P<sub>174</sub>, L<sub>176</sub> and E<sub>180</sub> essential for antibody recognition. Computational analysis was used to show that this epitope is located at an exposed domain of the VP2 capsid protein, readily accessible for immune recognition upon infection. Furthermore, this

peptide is also located in a region homologous to an immunoreactive domain in SV40 VP2 [27]. BLAST analysis of this peptide and subsequent experimental analysis of the obtained peptides showed that a number of alternative proteins might exist that could have elicited the observed immune responses. However, based on the limited geographical distribution of some of the organisms encoding these proteins and the intracellular localization of most of these proteins, it is unlikely that this would be the case, indicating that the immune responses should have been induced by infection with or exposure to JCPyV. The fact that there appears to be no correlation with JCPyV VP1 antibody levels, nor urinary viral load, indicates that these specific antibodies might be considered as a novel marker for infection with JCPyV.

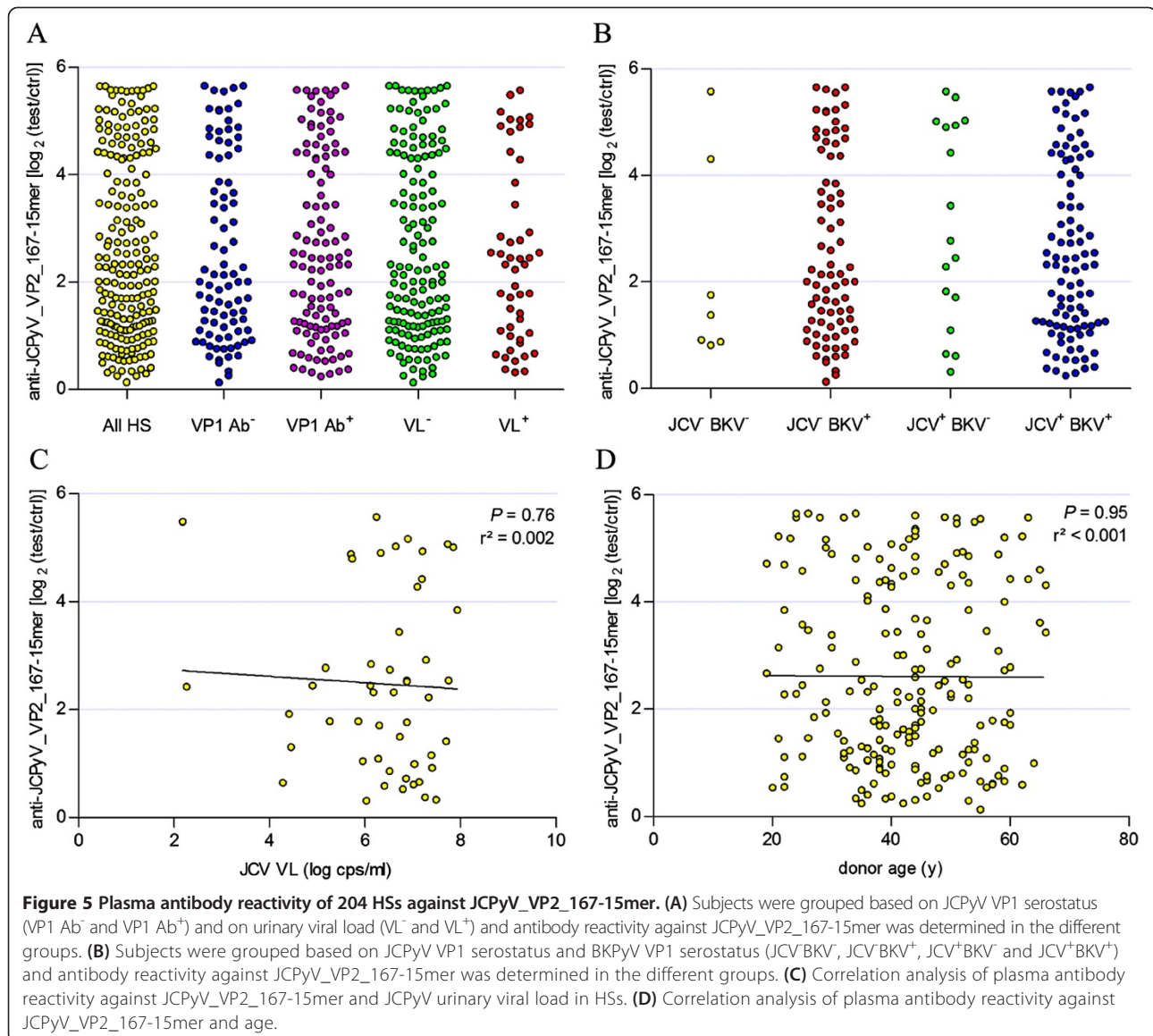
## Material and methods

### Ethics statement

The Ethics Committee [“Commissie voor Medische Ethiek - ZiekenhuisNetwerk Antwerpen (ZNA) and the Ethics committee University Hospital Antwerp] approved the Protocols, and Informed consents, which were signed by all subjects.

### Healthy subject samples

For the screening study a total of 50 healthy subjects were recruited in Belgium, for the evaluation study a total of 204 healthy subjects (HS) were recruited in Belgium [14,29,45]. The demographic description of the



HS populations is presented in Table 1. Plasma samples and urine samples were collected from all these HS and stored at  $-80^{\circ}\text{C}$  until further processing.

#### JC polyomavirus viral load assay

Analysis of the urinary viral load was performed as described previously [14].

#### JC polyomavirus VP1 serology assay

The anti-JCPyV antibody assay was performed as described earlier [45]. Samples were considered positive if OD values were higher than 2-fold the OD value of the blank sample (i.e.  $\log_2 \text{test}/\text{ctrl} > 1$ ).

#### BK polyomavirus VP1 serology assay

The anti-JCPyV antibody assay was performed identical to the method described for JCPyV VP1 with the exception that microtiter plates were coated with baculovirus expressed BKPyV VP1 capsid protein (Eurogentec, Belgium). Samples were considered positive if OD values were higher than 2-fold the OD value of the blank sample (i.e.  $\log_2 \text{test}/\text{ctrl} > 1$ ).

#### JC polyomavirus VP2 peptide microarrays

Peptide microarrays were performed as part of the work that was described earlier [29]. For this study only the peptides from JCPyV VP2 were included for further detailed analysis.

**Table 1 Overview of subjects investigated**

Variable	Screening study (n = 50)	Evaluation study (n = 204)
Gender, n (%)		
Male	22 (44%)	97 (48%)
Female	28 (56%)	107 (52%)
Age, median (Min-Max)	40.5 (23–59)	42 (19–66)
Race, ethnicity, n (%)		
White	38 (76%)	201 (98.5%)
Black	3 (6%)	0 (0%)
Asian	8 (16%)	2 (1%)
Other/unknown	1 (2%)	1 (0.5%)
JCPyV VP1 serology, n (%)		
Positive	36 (72%)	156 (57%)
Negative	14 (28%)	88 (43%)
JCPyV Viruria, n (%)		
Positive*	13 (26%)	50 (25%)
Negative	37 (74%)	154 (75%)
BKPyV VP1 serology, n (%)		
Positive	n.d.	178 (88%)
Negative	n.d.	24 (12%)
JCPyV_VP2_167-15mer serology, n (%)		
Positive	32 (64%)	165 (81%)
Negative	18 (36%)	39 (19%)

\*All JCPyV viruric subjects were part of the JCPyV VP1 seropositive subgroup.

### Molecular modeling of JCPyV\_VP2\_154\_36-mer

Molecular modeling was carried out using the program PEP-FOLD (web server <http://mobyli.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD>, [46,47]). The peptide sequence of JCPyV\_VP2\_154\_36-mer (GPSLFFSTI SQAFWNLVRDDLALTSQEIQRRQK) was submitted to the server for peptide folding and resulting structure was visualized using Jmol.

### Synthetic peptides

Biotinylated synthetic peptides were synthesized by standard procedures and purchased from JPT Innovative Peptide Solutions (Berlin, Germany Peptide serology assay). A list of all peptide sequences used in this study is provided in Additional file 2: Table S2.

### Peptide serology assays

For determination of peptide specific plasma antibody levels peptide ELISA was developed and set up as follows. Ninety-six well flat bottom plates (Nunc C96 Maxisorp, VWR) were coated with 100 µL of 1 µg/mL Streptavidin from *Streptomyces avidinii* (Sigma) in Dulbecco's PBS without calcium and magnesium (Gibco). The plates were incubated at 4°C for 16 hours. The plates were rinsed once with 200 µL PBS + 0.05% Tween-20 (washing

buffer) and blocked for 1 hour at 37°C with 200 µL of 10-fold diluted Blocker Casein (Pierce) in PBS (blocking solution). Upon removal of the blocking solution, plates were incubated with continuous shaking for 2 hours at room temperature with 100 µL of the selected biotinylated peptide, which were diluted at 1 µg/mL in blocking solution. The plates were rinsed 3 times with washing buffer, thereby eliminating unbound peptide. Then, the different wells were covered with 100 µL of human plasma samples, diluted 1:200 in blocking solution. Each sample was analyzed in duplicate. In control wells, blocking solution was added instead. The plate was incubated at 37°C for 1 hour. After incubation, a new triple rinsing cycle was repeated as described above. Then, the secondary antibody solution was added to each well. The solution contained an affinity purified Donkey anti-human IgG (H + L) peroxidase conjugate (Jackson ImmunoResearch) diluted 1:10,000 in blocking solution. The reaction mixture was incubated at 37°C for 1 hour. At the end of the incubation period, the plates were rinsed 3 times with washing buffer and treated with 100 µL SureBlue™ TMB Microwell Peroxidase Substrate (KPL). After 10 minutes of incubation the colorimetric reaction was stopped with 100 µL 1 N HCl. The plate was then read by the spectrophotometer (Spectra-Max, Molecular Devices) at a wavelength (λ) of 450 nm.



Samples were considered positive if OD values were higher than 2-fold the OD value of the blank sample (i.e.  $\log_2 \text{test}/\text{ctrl} > 1$ ).

### Statistical analysis

Differences in antibody levels between groups were assessed using a Mann-Whitney test. Differences between groups were considered statistically significant at  $P < 0.05$ . Correlation between different parameters was analyzed using linear regression.  $P$ -value was calculated to determine whether slope was significantly non-zero and strength of correlation was determined using  $r$ -value. All statistical analyses were performed using GraphPad Prism v 5.04.

### Additional files

**Additional file 1: Table S1.** Proteins identified upon BLAST analysis of JCV\_VP2\_171-11mer.

**Additional file 2: Table S2.** Sequences of peptides used in this study.

### Abbreviations

(PML): Progressive multifocal leukoencephalopathy; (JCPyV): JC polyomavirus; (BKPyV): BK polyomavirus; (MS): Multiple sclerosis; (ELISA): Enzyme-linked immunosorbent assay; (OD): Optical density; (HSs): Healthy subjects.

### Competing interests

Authors are current employees of Janssen Diagnostics BVBA or Janssen Research and Development, both being Johnson and Johnson Companies and may own stock or stock options in that company.

### Authors' contributions

OL and LJS designed the study setup. OL and TVL performed the experimental work. LJS performed the selection of peptides from the microarray data. OL performed the analysis of the data. LT and LJS contributed to revising the manuscript critically for important intellectual content and gave final approval of the version. All authors read and approved the final manuscript.

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