Diagnosing Polyomavirus Nephropathy without a Biopsy: Validation of the Urinary pyv-Haufen-Test in a Proof-of-Concept Study including Uromodulin Knock-out-Mice

Volker Nickeleit MD*, Dalton Butcher, Bawana D. Thompson, Lauraine H. Rivier, Harsharan K. Singh MD

The University of North Carolina School of Medicine at Chapel Hill, Department of Pathology and Laboratory Medicine, Division of Nephropathology, Chapel Hill, NC 27599 (USA)

Background: Polyomavirus nephropathy (PyVN) leads to kidney transplant dysfunction and loss. Since a definitive diagnosis requires an invasive kidney biopsy, a timely diagnosis is often hampered. In this clinical dilemma the PyV-haufen-test, centering around the detection of three-dimensional PyV aggregates in the urine, might provide crucial diagnostic information.

Methods: A multistep experimental design. Hypothesis: PyV-haufen form within the kidneys under high concentrations of uromodulin, a kidney specific protein; PyV-haufen are kidney-specific-disease-markers.

Results: Investigative step A showed colocalization of uromodulin with aggregated PyV (i) in ten kidneys with PyVN by immunohistochemistry, (ii) in urine samples containing PyV-haufen by electron microscopy/ immunogold labeling (n=3), and (iii) in urine samples containing PyV-haufen by immunoprecipitation assays (n=4). Investigative step B: In in-vitro experiments only high uromodulin concentrations of ≥ 1.25 mg/mL aggregated PyV, as is expected to occur within injured nephrons. In contrast, in voided urine samples (n=59) uromodulin concentrations were below aggregation concentrations (1.2 -19.6 µg/mL). Investigative step C: 0/11 (0%) uromodulin

*Correspondence to: Volker Nickeleit, MD, The University of N. Carolina School of Medicine, Department of Pathology and Laboratory Medicine, Division of Nephropathology, CB 7525, Chapel Hill, NC 27599-7525 / USA, Tel: ++ 9199187984, Email: volker_nickeleit@med.unc.edu

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DOI: 10.1093/infdis/jiae107
KO−/− mice with histologic signs of PyVN showed urinary PyV-haufen shedding compared to 10/14 (71%) WT+/+ mice.

Conclusion: PyV-haufen form within kidneys under high uromodulin concentrations. Thus, PyV-haufen detected in the urine are specific biomarkers for intra-renal disease, i.e. definitive PyVN.

Key words: biomarker, BK-polyomavirus, diagnosis, haufen-test, infection, kidney, polyomavirus nephropathy, transplantation, urine

BACKGROUND

In humans latent asymptomatic polyomavirus infections with BKPyV and/or JCPyV strains (PyV) are commonly found in the uro-renal tract. Viral disease with hemorrhagic cystitis or polyomavirus nephropathy (PyVN) are only seen under immunosuppressed conditions, such as post hematopoietic stem cell or kidney transplantation. So called definitive PyVN with end-organ disease is characterized by biopsy confirmed histologic evidence of lytic intra renal viral replication in tubules, tubular injury, tubulo-interstitial inflammation, fibrosis, nephron loss, and renal dysfunction [1-3]. It is mainly caused by the BK-virus strain (BKPyV) in kidney transplants with a prevalence of approximately 5% in western countries. Since specific antiviral treatment is lacking and therapy rests on the reduction of overall immunosuppression, definitive PyVN with end-organ injury is clinically feared with a graft loss rate of 8%-30% [2, 3].

To guide patient management, clinical risk assessment strategies for PyVN have been developed for kidney transplant recipients. They are based on laboratory assays detecting signs of BKPyV activation/replication by PCR in plasma and urine, that is, BKPyV-DNAemia/viremia and BKPyV-DNAuria/viruria. However, these screening tests only have limited predictive values to accurately diagnose definitive intra renal end-organ disease. Definitive PyVN can occur with low viremia levels and vice versa high viremia may not necessarily indicate viral nephropathy, such as seen post hematopoietic stem cell transplantation [4]. Thus, PCR assays are not “kidney disease specific” and cannot be reliably used to diagnose definitive PyVN [5-8].

Currently a diagnosis of definitive PyVN can only be rendered by a kidney biopsy. However, invasive biopsy procedures have limitations. Problems utilizing biopsies occur in patients with bleeding disorders and other contraindications preventing an invasive approach. Expensive biopsy work-up can also be challenging for underprivileged patients facing socio-economic problems. Furthermore, problems arise in pharmaceutical anti-PyV drug trials since renal biopsies are commonly not part of the study designs and patient cohorts may not be adequately stratified into those with versus those without definitive PyVN.

Thus, the inborn shortcomings of PCR based laboratory assays and the clinical limitations governing the use of invasive renal biopsies often hamper an optimal approach to patient management. Who has definitive PyVN and who does not?
We previously proposed a different method for diagnosing definitive PyVN. The so-called urinary PyV-haufen test (German for “heap” or “stack”) is not based on signs of PyV activation/replication but rather on specific structural urinary abnormalities, i.e. the detection of characteristic dense, three-dimensional PyV aggregates found in voided urine samples. These PyV-haufen are reportedly highly predictive for definitive PyVN [9-11]. However, the validity of the PyV-haufen test has not been fully established since it remains undetermined whether PyV-haufen are “kidney specific” markers.

The aim of the current study is to further validate the PyV-haufen test. We do so in a multi-step approach. We test the hypothesis whether uromodulin, a kidney specific glycoprotein also known as Tamm Horsfall Protein that is secreted in the loop of Henle, is a crucial component for the formation of PyV-haufen. Uromodulin as an essential requirement for PyV-haufen formation would link the pathogenesis and clinical significance of haufen to observations made with other intra renal casts, i.e. dense aggregates of cells or proteins that are shed into the urine. Such casts serve as important well-established diagnostic urinary markers in patients with kidney diseases.

In our current study we could prove that PyV-haufen detected in voided urine samples, are “cast-like” and, indeed, organ/kidney specific. They can serve as diagnostic biomarkers for intra renal PyV induced injury with lytic viral replication, that is definitive PyVN.

METHODS

The study was approved by the UNC Office of Human Research Ethics/Institutional Review Board (IRB # 05-1906, #08-1519, #20-0293) and the Institutional Animal Care and Use Committee (IACUC ID# 19-295.0). All rodent work was conducted with adherence to the NIH Guide for the Care and Use of Laboratory Animals. All research involving human subjects was conducted with adherence to the “Declaration of Helsinki” and the clinical and research activities being reported are consistent with the principles of the declaration of Istanbul as outlined in the “Declaration of Istanbul on Organ Trafficking and Transplant Tourism”. Urine and renal biopsy samples from humans were collected following local standard of care guidelines.

Study material

A histologic diagnosis of definitive PyVN was established (or excluded) at time of biopsy collection at UNC according to Banff recommendations [3]. In the current study, all cases included as definitive PyVN carried a diagnosis of PyVN in Banff disease class 2, pvl-scores 2 or 3 [3, 12]. Leftover formalin fixed and paraffin embedded tissue/biopsy material was used for additional studies as needed. In selected clinical cases, standard diagnostic EM analyses were conducted at time of allograft biopsy and archived electron micrographs were re-analyzed for current study purposes.
Voided urine samples were collected from three UNC kidney transplant patient cohorts at the time of diagnostic kidney biopsy (clinical biopsy indications: suspicion of rejection, acute kidney injury, drug toxicity, recurrent renal disease, PyVN, general signs of acute graft failure etc): (#1) patients with PyVN Banff disease class 2, pvl-score 2 or 3, viremia and viruria; (#2) patients with viruria/shedding of decoy cells, varying levels of viremia but no corresponding histologic/biopsy evidence of definitive PyVN, i.e. so-called PyV activators, and (#3) kidney transplant recipients without viruria/viremia and no histologic/biopsy evidence of definitive PyVN. In addition, for in-vitro experiments a few urine samples were collected from healthy non-transplant volunteers. The urine samples were either fresh frozen or fixed in 2% paraformaldehyde for storage at 4°C. The isolation of PyV/PyV-haufen followed established protocols including sequential centrifugation steps for clarification and concentration followed by negative staining and electron microscopic analysis (see supplementary data) [4, 9, 13, 14].

Black Swiss mice free of infection (Charles River Laboratories, Wilmington, MA) and uromodulin (Tamm-Horsfall) knock-out (KO) mice, strain 129/sv (kind gift of Dr Satish Kumar, MD and James M. Bates, University of Oklahoma Health Sciences Center, Oklahoma City, OK) were housed with access to food and water ad libitum. They were bred (Black Swiss outbred and 129/sv inbred) following general guidelines. Homozygosity in KO mice was monitored by genotyping and the absence of uromodulin further confirmed by ELISA on urine samples. All mice were infected by intra-peritoneal injection of 50 microliter murine polyomavirus strain A2 (containing 1-2x10^8 viral gene equivalents by PCR or 1x10^5 PFU (plaque forming units) per 50 microliter; A2 strain kindly provided by Prof. M. Fluck, Michigan State University) following a previously reported study design and protocol [15]. Infected mice were euthanized after 3-4 weeks when histologic evidence of intra renal lytic viral replication/PyVN had developed.

Urine from mice voiding more than 40 microliters was collected on parafilm and fixed in 2% paraformaldehyde for storage at 4°C. Subsequently, for EM grid preparation between 80 and 200 microliter fixed mouse urine samples were centrifuged at 1500 rpm for 5 minutes to clear debris. Thirty microliter fluid from the bottom of the Eppendorf tube were discarded while the remaining supernatant was used for further analysis. Negative staining and EM preparation to evaluate for the presence of murine polyomavirus/ murine polyomavirus haufen followed established protocols [4, 9, 14]. (For protocols on histology and immunohistochemistry, see supplementary data)

**Urinary pyv-haufen Definition**

In urine samples (human and mice), haufen were defined by EM as discrete aggregates of a minimum of six polyomaviruses with a typical surface structure forming unequivocal dense three-dimensional clusters (as reported previously) [9, 10].

DOI: 10.1093/infdis/jiae107
Urinary pyv-haufen are intertwined with Uromodulin: Analysis by Electron Microscopy and Immunogold Labeling

Three known PyV-haufen positive fresh frozen human urine samples were processed for EM analysis and immunogold labeling with an antibody directed against uromodulin using established protocols (see supplementary data).

Urinary pyv-haufen are intertwined with Uromodulin: Analysis by Immunoprecipitation and Western Blotting

PyV-haufen were isolated by centrifugation of four fresh frozen urine samples from four patients with previously established haufen shedding (patient cohort#1, see above). Centrifuged urine from patients without biopsy proven PyVN and previously confirmed lack of haufen shedding served as PyV-haufen negative control urine samples (n=4; patient cohorts #2, #3, and normal, see above). Dynabeads were coated with rabbit anti-SV40PyV-VP1 antibody at 37°C overnight. For negative-bead-control purposes, dynabeads were coated with rabbit IgG or they remained uncoated. Thirty microliters of urine concentrates were incubated with the coated beads and the eluates containing target proteins were subsequently saved. The immunoprecipitated protein complexes were separated by PAGE and transferred to Immobilon membranes. The membranes were immunostained with a mouse monoclonal anti-PyV-VP1 capsid antibody followed by an antibody directed against uromodulin. Chemiluminescence signals were captured on autoradiography films. PyV-capsid protein rendered a band in the 40-45 kDa and uromodulin in the 100-110 kDa range (see supplementary data for further details).

Uromodulin Concentrations govern pyv-haufen formation: an in-vitro Study

PyV, i.e. free virions, were collected from fresh frozen urine samples of twelve patients with viruria (BKPyV-DNAuria by PCR ranging between 6.3x10⁴ – 3.7x10⁶ gene equivalents/mL), previously EM confirmed urinary shedding of free virions without PyV-haufen, and no evidence of PyVN by renal biopsy (patient cohort#2, see above). Isolated free virions were resuspended in uromodulin spiked aqueous buffer solutions mimicking intra renal urine conditions in acutely injured tubules (test setting one; in mmol/L: Na 60, K 80, Ca 3.5, Mg 5, Cl 131 SO₄ 8, PO₄, Urea 47, Glucose 17; pH 6-0; as described previously) [16]. In parallel (test setting two) virions were resuspended in uromodulin spiked urine aliquots collected from non-transplant healthy volunteers (see above). The final uromodulin concentrations in both sample sets post spiking ranged from 0.31 mg/mL to 5.0 mg/mL. Negative controls were run in parallel with albumin replacing uromodulin at corresponding concentrations or with samples not spiked with protein. Post incubation PyV and PyV-haufen formation were evaluated by negative EM staining as previously described [10, 14]. In each sample PyV-haufen were recorded as either present or absent (see supplementary data for further details).

DOI: 10.1093/infdis/jiae107
Uromodulin Concentrations in voided human Urine Samples

Uromodulin concentrations were studied in 59 fresh frozen urine samples from patient cohorts 1-3 (see above) by sandwich ELISA technique using a modified protocol (see supplementary data) [17].

RESULTS

Histology of Polyomavirus Nephropathy: Intra-tubular pyv Aggregation and Uromodulin Colocalization

PyV undergo lytic replication in renal tubular epithelial cells leading to release of abundant virus progeny into injured tubular lumens where they tend to aggregate (Figure 1A). Such dense clustering of PyV in acutely injured nephron segments is associated with uromodulin colocalization. This phenomenon was studied in five human and five Black Swiss mouse kidneys with PyVN. In all nephrons showing intra-tubular viral aggregates, uromodulin was also noted (Figure 1B).

Urinary pyv-haufen are intertwined with Uromodulin: Analysis by Electron microscopy and Immunogold Labeling

PyV-haufen collected from voided urine samples showed aggregated virions intimately intertwined with uromodulin. This phenomenon was observed by electron microscopy and immunogold labeling of negatively stained urine samples (n=3 patients, Supplementary Figure 1).

Urinary pyv-haufen are intertwined with Uromodulin: Analysis by Immunoprecipitation and Western Blotting

PyV-haufen were isolated from urine samples of four patients with established PyV-haufen shedding by incubation with anti PyV-VP1 antibody coated magnetic Dynabeads. Samples from four patients without PyV-haufen shedding served as negative urine controls. Immunoprecipitated proteins were separated by gel electrophoresis and VP-1 and uromodulin visualized in western blots. Incubation of corresponding urine samples with either IgG coated beads or uncoated Dynabeads served as negative-bead-controls. Urine collected from all patients with PyV-haufen shedding showed strong co-migration of PyV-capsid protein 1 and uromodulin. No significant signals were seen in all controls (Figure 2).

Pyv Aggregation is promoted by high Uromodulin Concentrations: an in-vitro study

In the urine uromodulin concentrations and polymerization vary dependent upon location, i.e. bladder or intra renal nephron segments, and tissue integrity, i.e. normal or acutely injured tubules. Highest concentrations and extensive polymerization are found in the kidney and in injured nephrons.
We studied the effect of various uromodulin concentrations on PyV aggregation/haufen-formation in two in-vitro study settings (experimentally spiked samples): group#1 buffer conditions mimicking primary urine in injured nephron segments (ATI); group#2 normal voided urine samples from healthy volunteers (n=4 individuals, see above).

In both experimental conditions external uromodulin spiking resulted in PyV aggregation/haufen formation. In the setting of ATI (group#1) PyV aggregation was detected at uromodulin concentrations as low as 1.25 mg/mL and higher (Figure 3). In comparison, in normal urine samples (group#2) only highest uromodulin concentrations resulted in PyV aggregation, best seen at 5.0 mg/mL. No aggregation was noted with low uromodulin concentrations or in all control samples in which uromodulin was replaced with albumin (Table 1). The background baseline virion density was comparable in all samples tested based on the number of free virions detected by EM (18-25 free virions per EM grid square, medians; range: 10-30) indicating comparable test/PyV-aggregation conditions.

**Uromodulin Concentrations in voided Urine Samples**

Uromodulin concentrations were studied by ELISA in 59 voided urine samples from 42 patients: three patients (10 samples) with definitive biopsy proven PyVN (patient cohort# 1), 29 patients (29 samples) with PyV activation, i.e. viruria/viremia, but no PyVN by biopsy (patient cohort# 2), and 20 kidney transplant recipients (20 samples) with no viruria/viremia and no PyVN by biopsy (patient cohort# 3). Uromodulin concentrations in voided urine samples: definitive biopsy proven PyVN – 4.5 mg/mL (median, range 1.2-12.1); viremia/viruria but no PyVN - 4-6 mg/mL (median, range 0.5-17.1); no viremia/viruria and no PyVN - 7.3 mg/mL (median, range 3.5-19.6); all differences not significant.

**Uromodulin knock-out Mice with pyvn do not shed urinary pyv-haufen**

In order to investigate whether the formation and detection of PyV-Haufen in the urine is dependent upon the presence of intra renal/intra tubular uromodulin in-vivo, we studied uromodulin+/+ wild type (WT, n=14) and uromodulin−/− knock out (KO, n=11) mice [18] with definitive PyVN (Figure 4A). In rodents viral nephropathy with typical histologic signs of lytic replication can develop in 100% of young animals post infection (personal observation; manuscript in preparation).

PyV-haufen were not noted in any urine samples of knock-out mice (0/11) by EM. In contrast, urinary PyV-haufen were detected in 71% (10/14) WT mice (Figure 4B, Table 2). As expected in PyVN, all 25 mice (KO and WT) were shedding free polyoma virions in the urine as a positive “background control” for specimen adequacy [9, 14].

DOI: 10.1093/infdis/jiae107
DISCUSSION

Reliable non-invasive diagnostic biomarkers are much needed tools in the clinical repertoire, especially for diseases that commonly require invasive procedures for a definitive diagnosis. The diagnosis of definitive PyVN, as one example in this context, is conventionally based on histologic results from a kidney biopsy. PyV-haufen, i.e. three-dimensional tight aggregates of PyV found in voided urine samples, have previously been suggested as alternative non-invasive diagnostic biomarkers for definitive PyVN. However, further validation studies of the PyV-haufen test have never been conducted. Concern was raised regarding the organ specificity of the test. It was questioned whether haufen form within the kidneys or alternatively within the urinary bladder. Consequently, the clinical diagnostic use of the test was never generally endorsed.

In our current study we aim at proving the specificity of urinary PyV-haufen for intra renal end-organ injury caused by lytic PyV replication in tubules. We focus on a kidney specific protein called uromodulin or Tamm-Horsfall Protein that is known to be an essential component of general intra renal cast formation. Is uromodulin also crucial for the genesis of PyV-haufen and, consequently, can an intra renal origin of urinary haufen be proven?

We showed in a first set of experiments that PyV-haufen were closely intertwined with uromodulin. In histologic sections of definitive PyVN, densely aggregated virions were found in close proximity with uromodulin in injured tubules. This association was also demonstrated by EM and immunogold labeling experiments on PyV-haufen extracted from the urine and by immunoprecipitation and western blot studies: in haufen/an aggregated state PyV and uromodulin were tightly colocalized. In a second set of experiments, we demonstrated that PyV aggregate in-vitro only in high uromodulin concentrations ≥ 1.25 mg/mL and preferably in buffer conditions mimicking primary urine with high ionic strength as found in injured tubules [16]. In comparison, in voided urine samples collected from various patient cohorts and representing urine from the bladder, uromodulin concentrations were on average 100-1000 times lower than those required for PyV aggregation in the in-vitro studies (with overall uromodulin concentrations detected in our voided urine samples similar to a previous report [19]). These in-vitro observations argued against the genesis of PyV-haufen in the urinary bladder. In the third in-vivo set of experiments the intra renal origin of PyV-haufen was confirmed by utilizing uromodulin KO −/− mice with typical histologic features of a lytic intra renal PyV infection/PyVN. Uromodulin KO −/− mice with PyVN lacked urinary PyV-haufen shedding. In comparison, urinary haufen were found in 71% of uromodulin+/− WT mice with PyVN.

A discrepancy between previous studies on urinary PyV-haufen shedding (≥90% in humans with PyVN [4, 9]) and current rodent data (71% in WT mice) is caused by technical challenges collecting mouse urine.

Our experiments prove the intra renal genesis of PyV-haufen. For viral aggregation within tubules with lytic replication several promoting factors seem to act in concert: (i) high uromodulin
concentrations in the thick ascending limb of Henle/distal nephron segments where uromodulin is secreted, (ii) lytic PyV replication in distal nephron segments with high uromodulin concentrations, (iii) PyV induced acute tubular injury and corresponding low intra-tubular flow of primary urine with high ionic strength [16, 20, 21]. We demonstrate that, indeed, the chain of events for PyV-haufen formation is similar to other forms of cast formation, such as myeloma casts or various cellular casts [22-25] that are all well-established biomarkers of intra renal disease with uromodulin as a crucial building block. Consequently “cast-like” PyV-haufen found in voided urine samples serve as specific biomarkers for intra renal disease, that is definitive PyVN. PyV-haufen do not form in the urinary bladder.

Current recommendations for patients at risk for PyVN rest on the paradigm that individuals presenting with viral disease show laboratory signs of viral replication, such as viruria or viremia. However, vice versa, only a minority of patients with evidence of viral replication also present with end-organ-disease. Thus, diagnostic decision making is challenging since current clinical screening assays can identify patients at increased risk for end organ disease, but they cannot unequivocally allow for a definitive diagnosis of PyVN. Consequently, additional studies, such as invasive kidney biopsies are needed for diagnostic work-up (reviewed in [26]). Since the urinary PyV-haufen test is based on the detection of specific structural changes, that is the three-dimensional aggregation of PyV, it differs from “replication-based” assays. A qualitative PyV-haufen test in patient cohorts undergoing renal biopsy for various indications including rejection, drug toxicity, suspicion of PyVN etc and with varying levels of BKPyV-viremia reportedly had greater than 90% sensitivity, specificity, positive and negative predictive values for corresponding biopsy proven definitive PyVN [9]. There was no concern of urinary PyV-haufen shedding in patients with clinically insignificant latent PyV infections. A quantitative PyV-haufen analysis showed tight correlations with the overall degree of intra renal lytic PyV replication (Spearman’s \( \rho = 0.85 \)) and the Banff PyVN disease classes [4, 11]. (See supplementary data for more information on previously published clinical correlation studies and technical aspects of the PyV-haufen test). Thus, taking the current “proof-of-concept” data into consideration, the urinary PyV-haufen test can now be regarded as fully validated non-invasive biomarker for definitive PyVN.

When should the urinary PyV-haufen test be used in patient management (Figure 5). It is best suited as a targeted diagnostic tool for patients with signs of BKPyV replication based on currently recommended screening protocols, that is in an established risk group of patients. In this patient cohort the urinary PyV-haufen test may replace a kidney biopsy for the diagnosis of definitive PyVN. This is of special importance if renal biopsies cannot be easily collected, as seen in (1) patients with bleeding disorders, (2) some pediatric patients, or (3) those with socio-economic challenges, (4) in patients with a negative biopsy result but with concurrent and persistent signs of PyV replication/BKPyV-DNAemia requiring frequent repeat testing, or (5) in patients with persistent PyVN in order to monitor for signs of disease resolution [3, 8, 27, 28]. Patients facing socio-economic challenges and difficulties undergoing a costly biopsy procedure can especially benefit from a non-invasive urinary biomarker. In some patients kidney biopsies are marginal and
histologic results inconclusive. Here the PyV-haufen test can provide additional crucial diagnostic information. In addition, in pharmaceutical drug trials conducted to evaluate the efficacy of new antiviral agents, the test is of great diagnostic value since biopsy procedures are not commonly part of the trial designs. Consequently, study groups are often not adequately stratified into patients with versus those without definitive PyVN, and data analysis of the trial results might be challenging [7].

In conclusion, we contend that PyV-haufen in voided urine samples are specific structural biomarkers for intra renal lytic PyV replication, that is definitive PyVN. These biomarkers can be used to diagnose PyVN non-invasively, to improve diagnostic accuracy, and to enhance a personalized approach to patient management. Based on the current proof-of-concept study, the urinary PyV-haufen test is now further validated and should be incorporated into recommendations and guidelines for the management of transplant recipients at risk for PyVN.

**Acknowledgement:** All authors contributed to the study design, data interpretation, and preparation of the manuscript. The study was funded in its entirety by internal sources from the Department of Pathology and Laboratory Medicine/Division of Nephropathology at UNC/Chapel Hill/NC. No author reports any conflict of interest.

**Footnote Page:** The study was funded by internal sources from the Department of Pathology and Laboratory Medicine/Division of Nephropathology at UNC/Chapel Hill/NC. No author reports any conflict of interest. This work has not been published before except for partial presentation in abstract/poster form at the annual ‘American Society of Nephrology Meeting’ (San Diego, CA, 11/2012) and the ‘Banff Meeting on Allograft Pathology’ (Vancouver, Canada; 10/2015).

**References**


Figure 1A and 1B:

(A) Electron Micrograph: In PyVN in humans lytic viral replication in tubular epithelial cells results in the release of viral progeny into tubular lumens (T) where they tend to aggregate and form small clusters (arrows); injured tubular epithelial cell (E). Electron micrograph, 12,500x original magnification.

(B) Light Microscopy: In PyVN in humans lytic viral replication results in the intra tubular release of PyV progeny (stained in brown) that are seen in disintegrating epithelial cells as well as clustered in small aggregates in an extra cellular location. Within tubules virions are colocalized with uromodulin (stained in blue). IHC double labeling with antibodies directed against PyV-capsid protein (brown) and uromodulin (blue); light microscopy, 400x original magnification.
Figure 2:

Human Urine: Immunoprecipitation and Western Blots demonstrate tight colocalization of PyV-Capsid Protein and Uromodulin: (A) Test urine containing PyV-haufen (patients 1-4) - PyV-capsid protein 1 (PyV-VP1) was isolated by immunoprecipitation. Dynabeads were coated with an antibody directed against PyV-VP1. Immuno precipitated proteins (originating from the PyV-haufen) were subsequently separated by gel electrophoresis and visualized in western blots showing strong bands in the expected 40-45 kDa range for PyV-VP1 and 100-110 kDa for the colocalized uromodulin (uro), respectively. (B) Control urine: in urine samples containing PyV-haufen (patients 1-4), immuno precipitation with uncoated dynabeads or dynabeads coated with IgG followed by gel electrophoresis and western blotting did not reveal any specific signals for PyV-VP1 or uromodulin (uro). In addition, a control normal urine sample also did not show evidence of PyV-VP1 or uromodulin following immunoprecipitation with dynabeads coated with anti PyV-VP1 capsid antibodies.
Figure 3:

Negative Staining Electron Microscopy – in-vitro Experiment of PyV Aggregation: A uromodulin concentrations of 2.5 mg/mL promotes tight clustering of PyV in experimental buffer conditions mimicking primary urine compositions in acutely injured nephron segments. Transmission electron microscopy; 100,000x original magnification.

Figure 4A and 4B:

(A) Light Microscopy: A uromodulin−/− KO mouse with definitive PyVN. Murine PyV replication in tubular cells has resulted in widespread injury including nuclear enlargement and formation of type 4 (short arrows) and type 1 (long arrow) viral inclusions. Epithelial cell death and sloughing have resulted in segmental denudation of tubular basement membranes, that is virally induced ATI (arrow heads); (T= tubular lumen). Identical histologic changes are seen in corresponding uromodulin+/+ WT mice with definitive PyVN. Hematoxylin and Eosin stain; 200x original magnification.

(B) Negative Staining Electron Microscopy: A uromodulin+/+ WT mouse with definitive PyVN. In the urine a typical PyV-haufen with densely aggregated virions is found. Transmission EM, 100,000x original magnification.

DOI: 10.1093/infdis/jiae107
Figure 5:

PyVN risk assessment and diagnosis in kidney transplant recipients: flow chart illustrating recommendations for patient management including the urinary PyV-haufen test as fully validated non-invasive diagnostic biomarker.

Negative BKPyV-DNAemia/viremia by PCR assay: the differential diagnosis includes false negative PCR results due to mutant BKPyV strains, the presence of JCPyV or the presence of SV40PyV.

Note: other renal diseases can concur with PyVN.
PyVN SCREENING

CHECK VIREMIA TITERS

NEGATIVE
NO RISK FOR PyVN

POSITIVE
INCREASED RISK FOR PyVN

PLASMA: ≥ 4 log10 BK copies/mL
PRESUMPTIVE PyVN

RENEAL ALLOGRAFT
BIOPSY IF INDICATED

OR

URINARY PyV-
HAUFEN TEST

NEGATIVE
REPEAT URINARY
PyV-HAUFEN
TESTING

POSITIVE
DEFINITIVE PyVN

CLASSIFY DEFINITIVE PyVN FOR PROGNOSTICATION:
IN BIOPSY OR BY URINARY PyV-HAUFEN TEST
CLASS 1    CLASS 2    CLASS 3

PERSISTENT PyVN:
MONITOR VIREMIA AND / OR URINARY PyV-HAUFEN TEST

PyVN CLEARANCE:
NEGATIVE URINARY PyV-HAUFEN TEST
VARYING LOW LEVEL VIREMIA / NO VIREMIA
Table 1  Polyomavirus Aggregation under various Uromodulin Concentrations: an In-Vitro Study

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<th>Protein Concentration</th>
<th>Test Condition of Urine</th>
<th>PyV Aggregation/ Haufen Formation (yes/no)*</th>
<th>Number of free virions/grid square**</th>
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Uromodulin promotes PyV aggregation/haufen formation at high concentrations under ATI buffer conditions as seen in injured nephrons; even higher uromodulin concentrations are required for aggregation in normal urine samples. These in-vitro findings support an intra renal origin of PyV-haufen formation with a predilection along tubules with active PyV replication and associated tissue injury (ATI).

NA not applicable

Experiments were conducted in duplicate.

* evaluated in 25 grid squares/EM grid/test sample

** free non-aggregated virions were counted in 25 grid squares/EM grid; listed are median numbers per grid square (range: 10-30 free virions per grid square). The number of free virions was comparable in each test sample and served as an overall control parameter for equal viral density in the entire cohort.

### Table 2: Urinary PyV-haufen shedding in Uromodulin KO−/− and WT+/+ Mice with histologic evidence of PyVN

<table>
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<tr>
<th>Animal</th>
<th>Histologic Evidence of PyVN1 (yes/no)</th>
<th>Urine Samples</th>
<th>PyV-Haufen2 (present/absent)</th>
<th>Free Virions3 (present/absent)</th>
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**Wild Type**

**Uromodulin** $^{++}$

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DOI: 10.1093/infdis/jiae107
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KO – Uromodulin⁻/⁻ knock-out mice  
WT- wild type uromodulin⁺/+ mice

1 PyVN characterized by nuclear changes, lytic viral replication, acute tubular injury and focal inflammation (see Figure 4A).
2 Urinary PyV-haufen defined as 6 or more densely aggregated virions according to previous reports (see Figure 4B) [4, 10].
3 In PyVN voided urine samples also show the shedding of free, non-aggregated virions; this feature serves as internal non-diagnostic EM control for specimen adequancy as previously reported [14].