Determination of uromodulin in human urine: influence of storage and processing

Youhanna, Sonia; Weber, Julien; Beaujean, Viviane; Glaudemans, Bob; Sobek, Jens; Devuyst, Olivier

Abstract: BACKGROUND: Uromodulin (Tamm-Horsfall protein) is the most abundant protein excreted in the urine under physiological conditions. It is exclusively produced in the kidney and secreted into the urine via proteolytic cleavage. The involvement of UMOD, the gene that encodes uromodulin, in rare autosomal dominant diseases, and its robust genome-wide association with the risk of chronic kidney disease suggest that the level of uromodulin in urine could represent a critical biomarker for kidney function. The structure of uromodulin is complex, with multiple disulfide bonds and typical domains of extracellular proteins. METHODS: Thus far, the conditions influencing stability and measurement of uromodulin in human urine have not been systematically investigated, giving inconsistent results. In this study, we used a robust, in-house ELISA to characterize the conditions of sampling and storage necessary to provide a faithful dosage of uromodulin in the urine. RESULTS: The levels of uromodulin in human urine were significantly affected by centrifugation and vortexing, as well as by the conditions and duration of storage. CONCLUSIONS: These results validate a simple, low-cost ELISA and document the optimal conditions of processing and storage for measuring uromodulin in human urine.

DOI: [https://doi.org/10.1093/ndt/gft345](https://doi.org/10.1093/ndt/gft345)
Determination of Uromodulin in Human Urine:

Influence of Storage and Processing

Sonia Youhanna\textsuperscript{1}\textsuperscript{*}, Julien Weber\textsuperscript{1}\textsuperscript{*}, Viviane Beaujean\textsuperscript{3}, Robertus Glaudemans\textsuperscript{1}, Jens Sobek\textsuperscript{2}, and Olivier Devuyst\textsuperscript{1, 3}

\textsuperscript{1}Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich, Switzerland; \textsuperscript{2}Functional Genomics Center Zurich, Switzerland; \textsuperscript{3}Division of Nephrology, Université catholique de Louvain Medical School, Brussels, Belgium.

* SY and JW contributed equally to this study

\textbf{Running Title:} Uromodulin in human urine

\textbf{Correspondence:} to OD, Institute of Physiology, University of Zurich, Switzerland; E-mail: olivier.devuyst@uzh.ch
ABSTRACT

Uromodulin (Tamm-Horsfall protein) is the most abundant protein excreted in the urine under physiological conditions. It is exclusively produced in the kidney and secreted into the urine via proteolytic cleavage. The involvement of UMOD, the gene that encodes uromodulin, in rare autosomal dominant diseases, and its robust genome-wide association with the risk of chronic kidney disease suggest that the level of uromodulin in urine could represent a critical biomarker for kidney function. The structure of uromodulin is complex, with multiple disulfide bonds and typical domains of extracellular proteins. Thus far, the conditions influencing stability and measurement of uromodulin in human urine have not been systematically investigated, giving inconsistent results. In this study, we used a robust, in-house ELISA to characterize the conditions of sampling and storage necessary to provide a faithful dosage of uromodulin in the urine. The levels of uromodulin in human urine were significantly affected by centrifugation and vortexing, as well as by the conditions and duration of storage. These results validate a simple, low-cost ELISA and document the optimal conditions of processing and storage for measuring uromodulin in human urine.
INTRODUCTION

Urinary biomarkers constitute an essential tool for the diagnosis, classification, and prognosis of kidney diseases (1). Recent evidence pointed at uromodulin (originally named Tamm-Horsfall glycoprotein) as a potential urinary biomarker relevant for renal function, chronic kidney disease (CKD) and hypertension (2,3). Uromodulin is a 105 kD glycoprotein with seven N-glycosylation sites and a high-mannose chain. The protein contains 616 amino acids including 48 cysteine residues that are all engaged in the formation of disulfide bonds. Uromodulin contains three epidermal growth factor (EGF)-like domains and a zona pellucida (ZP) domain, found in many extracellular proteins, as well as a glycosylphosphatidylinositol (GPI)-anchoring site (3). Uromodulin is a kidney-specific protein, that is exclusively synthesized in the epithelial cells lining the thick ascending limb (TAL) of Henle’s loop (4). After proper trafficking and maturation in TAL-lining cells, uromodulin reaches the apical plasma membrane, to be cleaved and assembled in the urine as polymers forming a gel-like structure (5).

Uromodulin is produced at very high rate in the TAL, and is by far the most abundant protein in normal urine (excretion: 50-100 mg/day) (6). Functions attributed to uromodulin include protection against urinary tract infections; prevention of renal calculi formation by reducing aggregation of calcium crystals; and influencing transport processes by regulating the activity of NKCC2 and/or ROMK (7,8). Interest for uromodulin was re-ignited when it was discovered that mutations in the UMOD gene that codes for uromodulin are responsible for a series of monogenic disorders (familial juvenile hyperurecemia nephropathy, medullary cystic kidney disease type 2 or glomerulocystic kidney disease) all known as uromodulin-associated kidney disease (UAKD) (3). These disorders are characterized by severe tubulointerstitial damage, defective urinary concentration, hyperuricemia and gout, and progressive renal failure.
(9). The mutations often affect cysteine residues, resulting in conformational changes and intracellular aggregates of uromodulin. In turn, there is a strong decrease in the secretion of the protein by the TAL cells and a strong decrease in the urinary excretion of uromodulin (10-12). Lately, a number of genome wide association studies (GWAS) revealed that variants in the UMOD gene were strongly associated with markers of renal function and risk of developing hypertension and CKD in the general population (13-15). The association of uromodulin with both monogenic diseases and complex disorders such as CKD and hypertension provides a strong rationale for evaluating its urinary concentration as biomarker for renal function and CKD.

The determination of uromodulin in the urine is hampered primarily by its capacity to aggregate and the potential instability of such a complex protein. Documentation of sampling, processing and storage conditions is thus crucial for accurate uromodulin quantification. Despite the early characterization of antibodies specific for human uromodulin (16), the few reports on uromodulin dosage yielded conflicting results in terms of stability, storage conditions and processing of human urine (17-20). Important points such as the potential influence of urine centrifugation or vortexing, acidification or alkalinization, treatment with protease inhibitors, or normalization for urinary creatinine remain unsolved. Furthermore, earlier immunoassays were often based on poorly documented anti-uromodulin antibodies. Considering the increasing interest for a robust determination of uromodulin in the urine, the need for a high-throughput assay, and the limited and contradictory information available, we developed and characterized a robust ELISA for uromodulin and used this assay to investigate the stability of uromodulin under different treatment and storage conditions of human urine.
MATERIAL AND METHODS

Urine sample collection, storage and handling

Analyses were performed on second morning urine samples collected (mid-stream) in a sterile container from healthy volunteers aged 18-50 years, and processed within 2 h. This protocol was approved by the Ethical Committee of the Université catholique de Louvain.

The influence of human urine sample processing on the determination of uromodulin (Fig.1) was tested after vortexing the sample for 10 sec (Vortex-Genie 2, FAUST, Schaffhausen, Switzerland); centrifugation for 10 min at 3,600 rpm (Eppendorf Centrifuge 5430, Hamburg, Germany) at room temperature (i.e. standard protocol for urine processing and removing cells and debris; ref. 21); treatment with protease inhibitors (Leupeptin 1μmol/L; Sigma-Aldrich, St. Gallen, Switzerland; sodium azide 10 mmol/L); pH adjustment performed by drop titration with 1N HCl (to pH 2.0) or with 1N NaOH (to pH 8.0) using a Hanna HI 2211 pH meter; dilution using ultrapure deionized water (Destamat Bi 18E, QCS, Maintal, Germany) vs. TEA buffer (0.5% Triton X-100, 20 mM EDTA, pH 7.5). The effect of storage conditions was tested by comparing baseline levels with 1-week and 5-month storage at room temperature, +4°C and -20°C; 4-month and 8-month storage at -80 °C; five cycles of freezing-thawing (sample kept at -80 °C for 48 h followed by thawing on ice). Different sample sets were used to evaluate the influence of the various processing conditions as described.

Uromodulin ELISA

The in-house ELISA for uromodulin is a colorimetric based sandwich immunoassay using a sheep anti-human uromodulin antibody (Meridian Life Science, Memphis, USA; K90071C) as
the capture antibody. This antibody gives a single arc when tested by immuno-electrophoresis against fresh urine. The primary antibody was a monoclonal anti-human uromodulin antibody (Cedarlane Laboratories, Burlington, USA; CL 1032A) raised in mouse and validated in solid phase radioimmunoassay. The secondary antibody was a goat anti-mouse IgG (H+L) horseradish peroxidase conjugated (Bio-Rad, Cressier, Switzerland; 172.1011). The substrate was O-
Phenylenediamine dihydrochloride (OPD) (10mg/tablet) (Sigma-Aldrich). The OPD substrate solution was freshly prepared by dissolving a tablet in 25 mL of phosphate-citrate buffer [0.1 M citric acid monohydrate, 0.2 M Na₂HPO₄], pH 5.5. A volume of 5µL 30% H₂O₂ was added to 25 mL of substrate solution. Human uromodulin (Millipore, Billerica, USA) was used to establish the standard curve, with freshly prepared serial dilutions from the standard stock solution (100 µg/mL). Both the standard curve and a standard sample (uromodulin concentration 25 µg/mL) were systematically used for quality control (QC).

The determination of urinary uromodulin by ELISA was carried out as follows: a 96-well microtiter plate (NUNC MaxiSorp™, eBioscience, Vienna, Austria) was coated with 100 µL of 5 µg/mL capture antibody in coating buffer [500 mM H₃BO₃, 500 mM KCl, 345 mM NaOH, pH 9.0]. The plate covered with adhesive seal was incubated at 4°C overnight then washed three times with freshly prepared washing buffer (0.1 % Tween 20 in 10 mM phosphate buffer saline (PBS) pH 7.2 (PBS-Tween 0.1%)) using ImmunoWash 1575 Microplate Washer (Bio-Rad).

Unoccupied sites on the plate were blocked with 100 µL blocking buffer (0.5 % BSA in 10 mM PBS, pH 7.2) and incubated at 37°C for 1 h with a slow rotation (100 rpm). The plate was then washed three times with washing buffer and placed upside-down on absorbent paper to remove residual buffer. 100 µL of PBS-Tween 0.1 % was dispensed in all wells. Urine samples were stabilized at room temperature then diluted 1:50 in ultrapure deionized water, as preliminary
testing revealed no significant difference vs. dilution with TEA buffer (data not shown). A volume of 100 µL per well was distributed into the coated wells after vortexing. Standards and QC sample were run in duplicate whereas each urine sample was tested in 3 different dilutions. Deionized water was used as blank. After 1 h incubation at 37°C, the plate was washed three times and placed on absorbent paper. 100 µL of primary antibody diluted in PBS-Tween 0.1% (1µg/mL) was dispensed in each well; the plate was incubated at 37°C for 1 h (rotation, 100 rpm) then washed three times. The secondary antibody diluted 1:2,000 in PBS-Tween 0.1% was added to the wells for 45 min at 37°C and the plate washed three times. Color was developed by adding 100µL of OPD substrate solution. The plate was incubated at room temperature in the dark for 1 min, and the reaction stopped by adding 50µL of 2M H2SO4 to each well. Optical density (Infinite M200Pro, Tecan; Grödig, Austria) was read at 492nm and urinary uromodulin concentration was determined by referring to the standard curve. Uromodulin levels obtained using the in-house ELISA were compared to the commercial ELISA from MD Bioproduct (St. Paul, USA; M036020), following the protocol given by the manufacturer. This test has been used in several studies (13,22). Urinary creatinine levels (normalization) were measured using the Synchron® System Creatinine Assay (Unicell DxC Synchron®, Beckman Coulter, Brea, USA), following the manufacturer instructions.

Immunoblotting

Kidneys from Umod mice (23) were grounded in liquid nitrogen and homogenized as described previously (12). The homogenate was centrifuged at 1000 × g for 15 min at 4°C and the resulting supernatant at 100,000 × g for 120 min at 4°C. The pellet was suspened in homogenization buffer before determination of protein concentration (Pierce BCA protein assay kit; Thermo
Fischer Scientific; Rockford, USA). SDS-PAGE for mouse and human samples was performed under reducing conditions. Samples (20 μg of mouse and human kidney extract; 2μL of urine) were loaded after being mixed with Laemmli sample buffer and heated for 5 min at 95°C (kidney samples). Proteins were separated on 10 % SDS gel and transferred to nitrocellulose membrane for Western blotting. Membranes were blocked with 5 % milk blot for 30 min at room temperature then incubated overnight at 4°C with either sheep or mouse anti-uromodulin antibodies (1:400 in 0.5% BSA blocking buffer). Secondary antibodies were goat anti-mouse HPR conjugated (1:10,000) or polyclonal rabbit anti-sheep HRP conjugated (1:1,000), for 1 h at room temperature. Antigen-antibody reaction was detected by using ECL (Immun-Star HRP, Bio-Rad) and light-sensitive film (GE Healthcare, Glattbrugg, Switzerland). The molecular weight was estimated by running the Precision Plus Protein™ All Blue standard (Bio-Rad).

Deglycosylation and desialylation of uromodulin

Deglycosylation of uromodulin from human urine was carried out using PNGase F (PNGase F P0704S, New England Biolabs, Ipswich, USA) following the manufacturer’s protocol, whereas desialylation was performed according the protocol described by Parsons et al. (24). Briefly, uromodulin was precipitated from pooled human urine (1.5L) following the protocol of Tamm and Horsfall (2), dialyzed overnight at 4°C and then lyophilized (Virtis, Kloten Switzerland). Dry uromodulin was solubilized in 2.5M acetic acid (10 mg/ml), heated for 3 h at 82 °C, then washed 3 times with 15 mL PBS (pH 7.2) on Centicon (MWCO 30000) cartridge (Millipore). 1.5 μL of deglycosylated urine and 0.1 μL of desialylated uromodulin (vs. 0.5 μL of untreated urine) were loaded on 10 % acrylamide gel and analyzed as described above.

Immunohistochemistry
Colocalization of uromodulin with NKCC2 was carried out in cryosections of human and mouse kidney samples as previously described (10,12). Briefly, 5 µm-thick cryosections were blocked with 1% BSA/0.02% sodium azide-PBS for 30 min at room temperature, incubated for 2 h at room temperature with the sheep (1:400) or mouse (1:200) antibodies against human uromodulin, followed by washing and incubation with AlexaFluor633-conjugated donkey anti-sheep or goat anti-mouse (1:200) for 90 min at room temperature. Uromodulin-stained sections were then incubated with a polyclonal rabbit anti-NKCC2 antibody (Millipore; AB3562P; 1:100) for 3 h at room temperature, followed by washing and incubation with Alexafluor488-conjugated goat anti-rabbit antibodies (1:200). Sections were viewed on a Leica SP5 confocal microscope.

Surface plasmon resonance: Biacore

The interaction between uromodulin and the capture antibody was analyzed by surface plasmon resonance, using a Biacore T100 system (GE Healthcare, Uppsala, Sweden). Chemicals were from Sigma unless otherwise noticed. Binding experiments were performed in PBS buffer pH 7.4 containing 0.2% of Tween 20 at a flow rate of 30 µL/min at 25°C. Ultrapure and filtered water (“MilliQ”, Millipore, Billerica, USA) was used for preparing all solutions. The carboxymethyl dextran chip (CMD500L, XanTec bioanalytics, Düsseldorf, Germany) surface (1.2 mm² area) was cleaned before use by injecting 7 times a 50 mM NaOH solution containing 1 M NaCl for 30 sec at a flow rate of 5 µL/min. Surface binding is expressed in terms of changes in response units (RU) with 1 RU being approximately 1 pg/mm². Sheep polyclonal anti-uromodulin antibody (300 nM) in PBS-Tween was immobilized by amine coupling to the chip surface activated with
aqueous solutions of 0.4 M 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and 0.1 M N-
hydroxysuccinimide for 300 sec at 5 μL/min flow rate. For determination of kinetic constants, a
dilution series of four concentrations (19 nM, 39 nM, 78 nM, 156 nM) of uromodulin was
loaded on 10 % acrylamide gel and analyzed as described above.

Immunohistochemistry
injected using the T100 in multichannel mode. The reference channel used in parallel did not
contain immobilized antibody, in order to detect background response and unspecific binding of
analyte to the surface. Between two measurements, the surface was regenerated by injecting
twice 10 mM glycine at pH 2 for 30 sec, which completely removed uromodulin from the
antibody. For data evaluation, the measured sensogramms were referenced twice, first by
subtracting the signal from the reference channel, and second by subtracting the signal obtained
from injected pure buffer solution. Kinetic curves were evaluated using Biacore T100 Evaluation
Software (v. 2.0.2). A global fit was performed using the entire concentration series. Rate
constants for association and dissociation were calculated by taking a 1:1 binding model as a
basis.

Data analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 19 (IBM Corp.,
Armonk, USA). The Pearson correlation coefficient was used for correlation analysis, whereas
ANOVA and paired t-test were used for comparisons between the groups. A Bland-Altman plot
was used to evaluate agreement between uromodulin levels measured with the in-house ELISA
and commercial kit. Level of significance was set to p<0.05.
RESULTS

Characterization of the antibodies against human uromodulin

The antibodies used for the in-house ELISA were characterized by immunoblotting and immunostaining (Fig. 2). Immunoblot analysis of human urine and kidney samples in parallel with mouse kidney samples using the sheep polyclonal antibodies detected the uromodulin band at ~100 kD in all samples except the Umod KO kidney sample. The uromodulin band was also detected in human urine and kidney samples using the mouse monoclonal antibody (Fig. 2A, top panel). Both the polyclonal and monoclonal antibodies also appropriately identified the deglycosylated and desialylated forms of uromodulin (Fig. 2A, lower panels).

Staining of human and mouse kidney tissue samples with the mouse monoclonal or the sheep polyclonal antibodies detected uromodulin in the TAL, where it colocalized with NKCC2 at the apical surface area (Fig. 2B). Surface plasmon resonance (25) was further used to characterize the uromodulin – antibody interaction (Fig. 2C). As the isoelectric point of uromodulin (pI = 3.2) is too low for its immobilization to a carboxymethyl dextran surface, the capture sheep anti-uromodulin antibodies were immobilized at the surface of a sensor chip, and a dilution series of uromodulin was injected. For the interaction of immobilized sheep anti-uromodulin antibody with uromodulin, rate constants for association (k\text{on}) and dissociation (k\text{off}) of 4 x 10\text{4} M\text{\textsuperscript{-1}}sec\text{\textsuperscript{-1}} and 4 x 10\text{4} sec\text{\textsuperscript{-1}}, respectively, were determined, giving a dissociation constant K\text{D} (= k\text{off}/k\text{on}) of 10 nM. We also measured a strong binding response for the interaction of mouse anti-human uromodulin antibody to the sheep anti-uromodulin antibody - uromodulin complex. This situation
is comparable to the conditions in ELISA (see below). Regeneration of the surface with 10 mM glycine at pH 2.0 removed both the antibody and uromodulin.
Characteristics of the ELISA for uromodulin

When tested against purified human uromodulin, the in-house ELISA for human uromodulin showed a sensitivity (minimum amount of analyte which can be accurately detected) of 2.8 ng/mL and a linearity (correlation between concentration and optical density) of 1.0 (Fig. 3A). The inter- and intra-assay variabilities were determined at 3.28% and 5.46%, respectively. The assay had a detection range between 3.9 and 500 ng/mL. When compared with other assays, the in-house ELISA showed a wider range of measurement and lower intra- and inter-assay variability than commercially available routine kits (Table I). There was a robust correlation (r=0.905, p<0.001) when comparing the in-house ELISA with the MD Bioproduct kit. The Bland-Altman plot showed that the mean difference between both methods was -1.47 µg/mL (95% CI, -3.21 to 0.27 µg/mL) (Fig. 3B).

Influence of processing of urine samples

Since uromodulin has a tendency to aggregate, we first investigated the potential influence of vortexing and centrifugation on the determination of uromodulin levels in human urine (Table II). Comparison of fresh samples assayed before and after vortexing revealed a more than 50% increase in uromodulin levels (unindexed uromodulin: 5.02 ± 0.66 vs. 11.03 ± 1.67 µg/mL, respectively, p=0.001; indexed uromodulin: 10.84 ± 0.54 vs. 15.90 ± 1.45 mg/gr creat, respectively, p=0.001, n=37). Treating the urine samples with an usual centrifugation protocol (10 min, 3600 rpm) also showed a strong effect, since centrifugation yielded a significant decrease in unindexed (6.40 ± 0.63 vs. 13.27 ± 1.18 µg/mL, respectively, p<0.001) and indexed
Immunoblotting analyses (Fig.4) revealed that centrifugation was responsible for the precipitation of uromodulin in the pellet of cell debris. In comparison with the uromodulin band detected in fresh, non-centrifuged urine samples, the signal was strongly attenuated in the centrifuged urine sample while becoming apparent in the resulting pellet.

Alkalization of fresh urine sample to pH 8.0 did not influence the determination of urinary uromodulin, as compared with untreated (mean pH 5.68 ± 0.19) samples (unindexed uromodulin: 19.25 ± 4.14 vs. 20.60 ± 5.24 μg/mL, respectively, p=0.179; indexed uromodulin: 17.56 ± 2.28 vs. 18.34 ± 2.67 mg/gr creat, respectively, p=0.260, n=14). Likewise, acidification of urine samples to pH 2.0 did not result in a significant difference between values from untreated (mean pH 6.15 ± 0.59) samples (unindexed uromodulin: 10.01 ± 2.25 vs. 9.73 ± 2.07 μg/mL, respectively, p=0.621; indexed uromodulin: 18.86 ± 8.26 vs. 19.10 ± 8.55 mg/gr creat, respectively, p=0.782, n=8).

Influence of storage conditions

In order to cast light on the influence of storage conditions on the stability of uromodulin, we compared values obtained in samples analyzed at baseline and after 1 week or 5 months storage at room temperature, +4 °C and -20 °C. As compared to baseline, storage at either room temperature or 4°C or even -20°C were associated with decreased levels of both unindexed and indexed uromodulin (TableIII). Addition of protease inhibitors at time of collection had some
effect on the degradation of the samples conserved at -20°C, but not on those kept at +4°C. In any case, the addition of protease inhibitors was insufficient to prevent a significant decrease in the uromodulin levels as compared to baseline values. In contrast, 4-month storage at -80°C was not}

respectively, p=0.001, n=37). Treating the urine samples with an usual centrifugation protocol (10 min, 3600 rpm) also showed a strong effect, since centrifugation yielded a significant decrease in unindexed (6.40 ± 0.63 vs. 13.27 ± 1.18 µg/mL, respectively, p<0.001) and indexed
associated with significant changes in uromodulin levels in untreated samples. Further analyses revealed a slight but significant decrease after 8 months storage at -80°C (baseline uromodulin: 23.73 ± 1.57 µg/mL vs. 8-month: 20.13 ± 1.17 µg/mL, p=0.023, n=142). Freezing-thawing cycles (from -80°C to 0°C) showed no significant changes in the levels of urinary uromodulin as compared to baseline (Table IV).
DISCUSSION

Increasing evidence suggests that the level of uromodulin in urine could represent a useful biomarker for kidney function (3,26). In this study, we validated an efficient and cost-effective immunoassay and characterized the conditions of sampling and storage necessary to provide a faithful dosage of uromodulin in human urine. The urinary uromodulin levels were significantly affected by centrifugation and vortexing, as well as by the conditions and duration of storage.

To develop our in-house ELISA we used commercially available anti-uromodulin antibodies and validated their specificity in human and mouse kidney and urine samples. Both antibodies evidenced the ~100 kD band corresponding to uromodulin on Western blot, either in native or deglycosylated/desialylated state. They also showed the typical distribution along with NKCC2 in the apical membrane of the TAL. We used plasmon surface resonance to determine the binding constant for interaction of the immobilized sheep anti-uromodulin antibody to uromodulin to 10 nM which is in the expected range for an antibody-protein interaction. The immunoassay standard curve showed linearity over a broad range of values, allowing the detection of uromodulin with high sensitivity and very low inter- and intra-assay variability. It must be noted that, in contrast with previous results based on immunoblotting (20), dilution of the samples with deionized water yielded similar results than with TEA buffer. All these features, combined with an excellent correlation with the most used commercial ELISA, substantiate the interest of our immunoassay with the advantage of low cost, wide range of detection, and low variability.
Our analyses revealed a striking effect of vortexing and centrifugation on the determination of uromodulin in the urine. These two procedures yielded variations reaching 50% of the levels obtained on control, unprocessed samples (Table II). These findings are clinically
relevant, because low levels of urinary uromodulin have been suggested to be of diagnostic value in UAKD (3, 9-11). The effect of vortexing confirms the importance of the aggregation of uromodulin molecules in the normal urine. Uromodulin is also known to cofractionate with exosomes (27), the recovery of which is increased by vortexing (28). Uto et al. previously suggested that uromodulin may be trapped in cell debris or aggregated with crystals (18) after centrifugation protocols that are usual to remove contamination due to lysis or suspended cells (29). Our data confirm these findings and show that centrifugation of urine may decrease the level of uromodulin by ~30%. Thawed urine samples should thus be vortexed but not centrifuged before assaying uromodulin.

The question of the stability of uromodulin during different storage protocols is critical for analyzing large, multicentric cohorts. Previous studies based on small sample size yielded inconsistent conclusions about the influence of storage duration and temperature (17-20). Furthermore, these studies did not take into account normalization for urinary creatinine, which is usual for kidney biomarkers - at least in a stable situation (29,30). Our results, obtained on a large number of samples, reveal that short (1 week) and longer (5 month) storage at room temperature, 4°C or -20°C causes a significant decrease in indexed urinary uromodulin levels, largely due to decreased uromodulin. In contrast, a 4-month storage at -80°C is associated with marginal, non-significant decrease in the unindexed and indexed values. Of note, the decrease in unindexed uromodulin levels becomes significant after a 8-month storage at -80°C. The fact that storage of untreated urine samples at room temperature, 4°C or -20°C significantly decrease the level of uromodulin substantiates the observations of Kobayashi et al (20). This effect is only partially attenuated with protease inhibitors, which show some effect for samples kept at -20°C - but insufficient to prevent a significant degradation. Taken together, these data confirm the fact that urine samples should be stored at -80°C and analyzed within 3 months to give the most reliable
measurements. Of note, up to 5 freezing-thawing cycles on ice did not affect the stability of
uromodulin stored at -80°C.

Previous studies also reported inconsistent results in terms of treatments (detergents or
TEA buffer, alkalization) supposed to solubilize aggregates of uromodulin in urine (17-20, 31).
Some of these treatments may interfere with the binding of uromodulin to the ELISA capture
antibody (18). We verified here that dilution with deionized water gave similar results than with
TEA, and that urine alkalization (or acidification) had no effect on the determination of
uromodulin. These data support the conclusion that dilution of the sample with water before the
assay, combined with vortexing, is an efficient way of disaggregation (31).

In summary, these data indicate that reliable uromodulin measurements can be obtained
from untreated urine samples, provided they are immediately stored at -80°C and assayed within
3 months, with vortexing and dilution with water to prevent aggregation. This methodology will
be useful for high-throughput analyses of uromodulin and its validation as a biomarker for renal
function and risk of CKD.
attenuated with protease inhibitors, which show some effect for samples kept at -20°C - but insufficient to prevent a significant degradation. Taken together, these data confirm the fact that urine samples should be stored at -80°C and analyzed within 3 months to give the most reliable
ACKNOWLEDGEMENTS

These studies were supported in part by an Action de Recherche Concertée (ARC, Communauté Française de Belgique); the FNRS and FRSM; the Inter-University Attraction Pole (IUAP, Belgium Federal Government); the NCCR Kidney.CH program (Swiss National Science Foundation); the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 246539 (Marie Curie) and grant n° 305608 (EUREOmics) and the Gebert Rüf Stiftung (Project GRS-038/12). The expert assistance of N. Amraoui, H. Debaix, S. Druart, Z. Guo and S. Terryn is appreciated. We thank Dr. L. Rampoldi for providing the kidney extract obtained from the Umod KO mouse developed by Prof. X-R. Wu (Ref. 21), and Dr. S. Schauer for helpful discussions about the Biacore experiments.

The authors declare no competing financial interests.
REFERENCES


Table I. Comparison of the characteristics of the in-house ELISA for uromodulin and the available commercial ELISA kits.

<table>
<thead>
<tr>
<th>Kit</th>
<th>Detection range (standard curve)</th>
<th>Inter-assay Variability</th>
<th>Intra-assay Variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-house</td>
<td>3.9 - 500 ng/mL</td>
<td>3.28%</td>
<td>5.46%</td>
</tr>
<tr>
<td>MD Bioproduct</td>
<td>2.34 - 150 ng/mL</td>
<td>11.63%</td>
<td>8.36%</td>
</tr>
<tr>
<td>(Cat. M036020)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BioVendor</td>
<td>0.5 - 32 ng/mL</td>
<td>6.4%</td>
<td>2%</td>
</tr>
<tr>
<td>(Cat. RD191163200R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USCN Life Science Inc.</td>
<td>3.13 - 200 ng/mL</td>
<td>&lt;12%</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>(Cat. E96918 Hu)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table II. Effect of sample processing (vortex, centrifugation) on the concentration of uromodulin in the urine.

<table>
<thead>
<tr>
<th></th>
<th>Unindexed Uromodulin (µg/mL)</th>
<th>Indexed Uromodulin (mg/gr creat)</th>
<th>P</th>
<th>P</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vortex</td>
<td>11.03 ± 1.67</td>
<td>15.90 ± 1.45</td>
<td>0.001</td>
<td>0.001</td>
<td>37</td>
</tr>
<tr>
<td>No vortex</td>
<td>5.02 ± 0.66</td>
<td>10.84 ± 0.54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centrifugation</td>
<td>6.40 ± 0.63</td>
<td>9.97 ± 1.43</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>53</td>
</tr>
<tr>
<td>No centrifugation</td>
<td>13.27 ± 1.18</td>
<td>15.66 ± 1.34</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Urine samples were vortexed for 10 sec. Centrifugation was performed for 10 min at 3600 rpm at room temperature. Two different sets of samples were used to test the influence of vortexing (N=37) and centrifugation (N=53).
Table III. Effect of storage conditions (duration, temperature, protease inhibitors) on the concentration of uromodulin in the urine.

<table>
<thead>
<tr>
<th>Storage Duration</th>
<th>Baseline (μg/mL)</th>
<th>P</th>
<th>Indexed (mg/gr creat)</th>
<th>P</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week storage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>12.39 ± 2.41</td>
<td>P</td>
<td>22.70 ± 3.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>6.14 ± 1.34*</td>
<td></td>
<td>13.00 ± 2.34*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+4 °C</td>
<td>7.22 ± 1.60*</td>
<td>0.078#</td>
<td>11.49 ± 1.50*</td>
<td>0.014#</td>
<td>13</td>
</tr>
<tr>
<td>-20 °C</td>
<td>9.98 ± 1.96*</td>
<td></td>
<td>18.69 ± 3.18*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 month storage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>36.37 ± 2.62</td>
<td>0.354</td>
<td>24.05 ± 1.26</td>
<td>0.412</td>
<td>61</td>
</tr>
<tr>
<td>-80 °C</td>
<td>35.47 ± 2.32</td>
<td></td>
<td>23.30 ± 1.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 month storage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>28.50 ± 6.76</td>
<td></td>
<td>26.48 ± 3.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+4 °C</td>
<td>10.17 ± 3.96*</td>
<td>0.068#</td>
<td>10.27 ± 2.35*</td>
<td>0.001#</td>
<td>10</td>
</tr>
<tr>
<td>-20 °C</td>
<td>16.52 ± 5.08*</td>
<td></td>
<td>15.78 ± 2.73*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+4 °C &amp; PI</td>
<td>11.04 ± 4.69*</td>
<td></td>
<td>10.80 ± 2.32*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-20 °C &amp; PI</td>
<td>20.23 ± 5.27*£</td>
<td>0.111#</td>
<td>18.71 ± 2.69*£</td>
<td>0.003#</td>
<td></td>
</tr>
</tbody>
</table>

PI, treatment with protease inhibitors (Leupeptin and sodium azide). Three different sets of urine samples were used to assess influence of storage after 1 week (N=13), 4 months (N=61) and 5 months (N=10) vs. baseline levels. * p < 0.05 storage condition vs. baseline, £ p < 0.05 no vs. protease inhibitors, paired t tests; # ANOVA
Table IV. Effect of freezing-thawing cycles on the concentration of uromodulin in the urine.

<table>
<thead>
<tr>
<th>Freezing-thawing cycles (-80°C to 0°C)</th>
<th>Unindexed Uromodulin (µg/mL)</th>
<th>P</th>
<th>Indexed Uromodulin (mg/gr creat)</th>
<th>P</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>22.96 ± 4.75</td>
<td></td>
<td>18.14 ± 2.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22.01 ± 3.81</td>
<td></td>
<td>20.19 ± 3.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>24.74 ± 4.05</td>
<td>0.616#</td>
<td>23.67 ± 3.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16.74 ± 2.68</td>
<td></td>
<td>18.17 ± 2.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>16.96 ± 3.36</td>
<td></td>
<td>16.20 ± 1.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>19.42 ± 4.56</td>
<td></td>
<td>15.91 ± 2.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# ANOVA