Day-to-Day Variation of the Urine Protein: Creatinine Ratio in Female Dogs with Stable Glomerular Proteinuria Caused by X-Linked Hereditary Nephropathy

Mary B. Nabity, May M. Boggess, Clifford E. Kashtan, and George E. Lees

Background: Interpretation of serial urine protein: creatinine (UPC) values is confounded by a lack of data regarding random biologic variation of UPC values in dogs with stable glomerular proteinuria.

Hypothesis: That there is minimal day-to-day variability in the UPC of dogs with unchanging proteinuria and the number of measurements needed to reliably estimate UPC varies with the magnitude of proteinuria.

Animals: Forty-eight heterozygous (carrier) female dogs with X-linked hereditary nephropathy (XLHN) causing stable proteinuria.

Methods: Urine samples were obtained daily by cystocentesis for 3 consecutive days on 183 occasions (549 samples). The UPC was measured for each sample with a single dry-film chemistry auto-analyzer. Data were analyzed retrospectively by a well-defined renal disease causing a stable magnitude of proteinuria and the number of measurements needed to obtain a reliable estimate of the actual UPC ratios.

Results: To demonstrate a significant difference (P < .05) between serial values in these proteinuric dogs, the UPC must change by at least 35% at high UPC values (near 12) and 80% at low UPC values (near 0.5). One measurement is adequate to reliably estimate the UPC when UPC < 0.4, but 2–5 determinations are necessary at higher UPC values.

Conclusions and Clinical Importance: These guidelines for interpretation of serial UPC values in female dogs with XLHN may also be helpful for interpretation of UPC values in dogs with other glomerulopathies.

Key words: Creatinine; Power of mean model; Renal disease; Urine; Variance components.

The urine protein-to-creatinine ratio (UPC) has become widely used in veterinary medicine as an index of magnitude of proteinuria in dogs in the 2 decades since studies first validated its use for this purpose. A UPC persistently ≥ 0.5 is indicative of an abnormal degree of proteinuria, whereas a UPC < 0.5 is consistent with absence of significant proteinuria. Greater magnitudes of proteinuria correlate with severity and progression of renal disease in humans and animals, including dogs. In addition, recent studies have shown that various therapeutic interventions, such as administration of angiotensin converting–enzyme inhibitors or dietary modifications, can reduce magnitude of proteinuria and slow progression of renal disease. Because of such findings, veterinarians have begun to recognize potential benefits of reducing proteinuria in dogs with a variety of renal diseases, and treatment of dogs to reduce the magnitude of their proteinuria has been recommended. Serial UPC monitoring is used to assess treatment efficacy, disease progression, and prognosis in proteinuric dogs. Many important clinical decisions for dogs with proteinuria hinge on an ability to detect clinically important changes in serial UPC values.

Determining whether a UPC value has changed on serial measurements requires knowledge of the variability of the UPC, when the magnitude of proteinuria is unchanging. To the authors’ knowledge, there are no published reports of day-to-day biologic variability of the UPC in proteinuric dogs (UPC ≥ 0.5). One reason for this lack of published reports is that a wide range of diseases can cause proteinuria in dogs, and the majority of these diseases have a variable effect on the progression of proteinuria. Thus, random biologic variation cannot be easily distinguished from other sources of variation. In this study, we used dogs with a rare but well-defined renal disease causing a stable magnitude of glomerular proteinuria for extended periods of time in order to assess the random day-to-day fluctuation in the UPC ratio.

The goals of this study were to establish guidelines for the expected variability in the UPC of dogs with unchanging proteinuria and the number of measurements needed to obtain a reliable estimate of the actual UPC value.

Materials and Methods

Dogs

Retrospective analysis was performed of data collected between January 1999 and October 2002 from 48 young-adult (12–32 months old) heterozygous (carrier) female dogs with X-linked hereditary nephropathy (XLHN). The dogs were members of a single family maintained in a colony at Texas A&M University since 1997. XLHN in this kindred is caused by a nonsense mutation in the COL4A5 gene that encodes the α5 chain of type IV collagen, which is a crucial component of normal glomerular basement membranes (GBM). The salient clinical and pathologic features of the nephropathy that occurs in dogs with this gene defect have been described. In carrier females, these features include mosaic expression of type IV collagen peptides that are normally found in
the GBM and onset of persistent glomerular proteinuria between 3 and 6 months of age.

All puppies produced in the colony were raised with a standardized protocol for feeding, husbandry, routine health care, and socialization. In addition, renal function of all carrier females was monitored by measuring serum creatinine concentration every 3 months. A few (<10%) carrier females had intermittent or persistent increases in serum creatinine concentration before 3 years of age, but the great majority (>90%) of the carrier females raised within the colony remained clinically healthy and maintained good renal function as adolescents and young adults. Despite their proteinuria, these dogs had urine specific gravity values indicative of adequate urine concentrating ability (>1.035) and stable serum creatinine concentrations in the middle of the reference range. Forty-eight of the young adult XLHN carrier females having these attributes were selected for the studies of proteinuria that generated the data retrospectively analyzed for this report.

During studies, dogs were housed in runs in a temperature-controlled room with a 12-hour light-dark cycle, and they were fed once daily in the afternoon. Dogs were leash walked outside or were permitted short periods of unrestrained access to an exercise area daily.

**Data Collection**

Several studies were conducted in a similar fashion to examine the influence of various factors on magnitude of proteinuria in dogs with glomerular disease. The study protocols were reviewed and approved by the Texas A&M University Laboratory Animal Care Committee, and results of the studies have been published or reported at scientific meetings. Magnitude of proteinuria was assessed in each dog by measuring the UPC once daily on each of the last 3 days of various periods during which the dog was maintained or treated in a specified manner. All dogs were evaluated after 1, 2, or 4 weeks without treatment. In addition, some studies included treatments that were given for periods of 4 or 6 weeks, during which the dogs were evaluated on the last 3 days of each successive 2-week interval. After treatment (being fed a specified diet or given prednisone), dogs were either crossed over to a second diet or observed after cessation of prednisone administration for an additional 4- or 6-week period, during which evaluation at 2-week intervals continued. Changes in the UPC typically occurred sufficiently quickly after changes in treatments, so that mean values had stabilized by 2 weeks after initiation or cessation of treatment. An aliquot of urine obtained by cystocentesis was submitted for quantitative aerobic bacterial culture to verify absence of urinary tract infection in each dog at the outset of her study protocol and again at the end of her protocol if she was studied for >1 week.

The combined data available from the 48 dogs used in these studies included a total of 183 3-day evaluation periods and 549 UPC determinations. All 48 dogs were evaluated 1 to 2 times before treatment, resulting in 75 3-day evaluation periods (225 UPC determinations) without treatment. Twelve dogs were treated with a high- or low-protein diet, resulting in 84 3-day evaluation periods (252 UPC determinations) during the 14-week treatment period. Six dogs were treated with prednisone (2.2 mg/kg PO q24h), resulting in 24 3-day evaluation periods (72 UPC determinations) during the 8-week treatment and posttreatment periods.

**Sample Collection and Assay**

All urine samples were collected by cystocentesis during the morning (ie, before the dogs were fed each day). Samples were refrigerated immediately after collection and assayed within 6 hours. Urine was centrifuged (300 × g for 5 minutes), and urine protein and creatinine concentrations in the supernatant were measured with a dry-film chemistry auto-analyzer. Proteinuria (mg/dL) was determined by a colorimetric method with a pyrocatechol violet-molybdate complex. Urine creatinine (mg/dL) was determined by a colorimetric enzymatic method with creatinine amidohydrolase. Samples were diluted by the instrument according to the manufacturer’s specifications when necessary.

**Analytical Variability**

Analytical variability of UPC was determined by means of 3 urine samples, one each having a low (0.5), moderate (2.7), or high (7.9) UPC. Each of these samples was measured 6 times over 72 hours (once in the morning and once in the afternoon of each day) to assess interassay variability. Each sample was also measured 10 consecutive times during a single instrument run to determine intraassay variability. Samples were stored at 4°C between analyses. Assays were performed for only 3 days because urine protein determinations may be unreliable after more than 3 days of sample storage at 4°C according to the instrument guidelines, and freezing may precipitate proteins. Analytical variability was also assessed with control standards that were measured each day as UPC measurements were performed on clinical patients over the course of 1 month. The creatinine concentrations of the control standards were comparable to those of the urine samples; however, the protein concentrations of the control standards were much lower than those of the moderate- and high-UPC urine samples, resulting in much lower overall UPC values for the control standards than for the canine urine samples used to assess analytical variability.

The variance components for analytical variability were estimated by 3-way random effects analysis of variance (ANOVA). By means of the representative low, moderate, and high UPC urine samples, the estimated intra-assay standard deviation = 0.07 and intraclass correlation coefficient (ICC) = 0.99965 (0.99895, 1.00036), and interassay standard deviation = 0.06 and ICC = 0.99973 (0.99915, 1.0003). By means of the control standards, the estimated interassay standard deviation = 0.03, and the ICC = 0.99374 (0.97611, 1.01136). Compared with biologic variability, analytical variability is optimally low, and we assume that its contribution to total variation is minimal. Thus the analytical variability was not incorporated separately into the model but was incorporated into the between and within evaluation variance components described in the following section.

**Statistical Methods**

The 2 major sources of variation are biologic variation and analytical variation. The biologic variation includes that which is due to intra-individual and interindividual variation (within and between evaluation variance, respectively). The within-evaluation variance component (σe2) reflects the variability due to repeated measurements on the same dog within a given evaluation period (3 consecutive daily measurements). σe was modeled as a function of mean UPC by σe = σ × UPC, also known as a power of the mean model. This model was chosen because the standard deviation (σe) of the UPC measured over 3 days increases with higher UPC values in all dogs, whether untreated or treated, and is therefore not constant with respect to the UPC level (Fig 1). If the variability of the UPC had been constant over the full range of values, then it could have been readily estimated with a straightforward technique (eg, random effects ANOVA). However, variation that increases in proportion to the mean is a common phenomenon with values whose lower limit is bounded by zero, which is the case with serum and urine chemistry analytes. This requires the use of other methods to estimate variance.
The between-evaluation variance component ($\sigma^2$) reflects the variability due to measurements obtained from different evaluation periods, as a result of measurements obtained over a period of weeks to months, either from the same dog or from different dogs. This component was modeled with a random effect assuming a normal distribution for the 3-day evaluation period. Since the sample contained multiple evaluations performed on the same dog, bootstrapped standard errors were used to take into account the possible correlation. All estimations were performed with Stata 9.

The estimate of the standard deviation ($s_w$) is denoted as $s_w$; similarly, the estimate of $\sigma_w$ is denoted as $s_w$.

The estimate of the standard deviation, $s_w$, was used to calculate the reference change value (RCV): $RCV = 1.96 \times (\bar{s}_w)^{1/2} = 2.77 \times s_w$. The RCV represents the amount of change that is reasonably explained by biologic variation. Therefore, if no change in the magnitude of proteinuria has occurred, serial measurements should fall within the interval created by adding or subtracting the RCV from the original UPC, with 95% confidence. The reference change value percentage (RCV%) was also determined: $RCV% = \frac{RCV}{UPC} \times 100$. This value is the percentage change in the UPC necessary to determine an increase or decrease in UPC.

The RCV and RCV% are meant for comparison of 2 single UPC measurements. However, the RCV can be extrapolated to include multiple averaged UPC measurements. If the UPCs from several urine samples have been averaged in order to obtain a more accurate estimation of the UPC, then the RCV can be calculated by: $2.77 \times (s_w/n)$, where $n$ is the number of averaged measurements and $s_w$ is their standard deviation.

The ICC = $\frac{\sigma^2}{[\sigma^2 + \sigma^2_w]}$. This reflects the reliability of repeated measurements, so that a higher ICC is suggestive of a more reliable measurement.

The Spearman-Brown prediction formula relates the ICC to the number of measurements (k) required to achieve a given reliability: $ICC^k = ICC[1 + ((k-1) \times ICC)]$. Acceptable reliability was set at $ICC^1 = 0.9$.

The standard error for the quantities described above were calculated by the delta method.

Because UPC measurements were obtained from some dogs that were given treatments (prednisone and varying diets), the effect of treatment on variance of UPC was analyzed. Treatment effect on variance was determined by comparing the $s_w$ of the untreated evaluations to the $s_w$ of each treatment group. The 95% confidence intervals for the $s_w$ of each treated group largely overlapped with those for the untreated group supporting the absence of treatment effect on the variability of the UPC (not shown). Therefore, all observations were pooled over treatments for the analysis.

**Results**

The standard deviation estimates for UPC day-to-day variability were $s_w$: $0.24 \times UPC^{0.74}$ and $s_b$: $2.01 (1.78, 2.23)$. Normality of the residuals was verified by the use of a normal probability plot.

Applying the $s_w$ results, $RCV = 2.77 \times (0.24 \times UPC^{0.74})$ and $RCV% = [2.77 \times (0.24 \times UPC^{0.74})]/UPC \times 100$. The RCV and RCV% were then plotted against the UPC value to provide continuous plots that can be used to identify significant changes from an initial UPC value between 0.5 and 12 (Figs 2, 3). For example, to be 95% confident that the magnitude of proteinuria has increased from a baseline UPC of 5, then with the graphs, a subsequent UPC value must increase by at least 48% or 2.4, resulting in a value of 7.4. By adding and subtracting the RCV from the initial UPC, the critical values can be determined that indicate the largest possible deviation from baseline that can be reasonably expected due to random biologic variation. Subsequent measurements above and below these critical values are far enough from baseline to support a significant change in the UPC (Table 1). To summarize, at low UPC values (near 0.5), a minimum change in the UPC of 80% is required to demonstrate a significant difference ($P < .05$) in serial values, whereas at high UPC values (near 12), a minimum change of 35% is necessary.

The ICC and the Spearman-Brown prediction formula were used to estimate the number of averaged UPC determinations needed to obtain a reliable estimate of the UPC. Results revealed that separate urine samples give very similar results at low UPC values; however, results from separate urine samples vary more widely at larger UPC values. Table 2 lists the number of UPC determinations needed to obtain an estimated ICC ≥0.9 for various UPC measurements. In general, one measurement is adequate to estimate the UPC when
values are $\leq 4$. An average of 2–3 UPC determinations is necessary when the UPC is 4–8, and 4–5 averaged measurements are necessary when the UPC is $> 8$ to adequately estimate the true UPC value.

**Discussion**

Most of the carrier female dogs with XLHN raised within the colony remained clinically healthy and maintained good renal function as young adults (1–5 years of age). In addition, their prevailing magnitude of proteinuria as estimated by determining their UPC value averaged over a 3-day period (in order to minimize the confounding effects of random day-to-day variation) remained stable from week to week and month to month. During such long periods of time with little or no evidence of any substantial change in renal disease severity, most or all of any short-term (day-to-day, week-to-week, or month-to-month) change in UPC value could be attributed to random biologic variation or to effects of treatments on the prevailing magnitude of proteinuria rather than to a fundamental change in the severity of the underlying primary disease. The validity of this assumption was supported by the results of studies that used crossover experimental designs to investigate the influence of various treatments on the magnitude of proteinuria in these dogs. When dogs in such experiments returned to treatments or conditions under which they initially had been evaluated weeks or months previously, their proteinuria consistently returned to the magnitude that had been observed before. That rationale also underlies the use of data from those studies to assess biologic variation of the UPC in this report. Because of the slowly progressive nature of the disease in these dogs and the lack of evident treatment effect on variance, most or all of the day-to-day variation in the UPC observed during 3-day evaluation periods was reasonably attributed to random biologic variation rather than to fundamental changes in magnitude of proteinuria because of altered disease status or treatment effect.

The development of the appropriate statistical model to use in this study was challenging. Coefficient of variation (CV) has historically been used in the medical field to express the variability of an analyte, and CV was used in most of the studies that address UPC variability in humans. However, this method does not allow for the separation of total variance into components. Separation into variance components is necessary because it allows a statistical model to be developed that predicts the expected variation of the UPC over a range of values. In addition, a model that could account for unequal variance was required. In the present study, maximum likelihood combined with the power-of-mean model allowed for calculation of the

**Table 1.** Subsequent UPC values required to demonstrate a significant (95% confidence) decrease or increase in UPC following an initial determination.

<table>
<thead>
<tr>
<th>Initial UPC</th>
<th>Value required to demonstrate significant decrease</th>
<th>Value required to demonstrate significant increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>&lt;0.1</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>1</td>
<td>&lt;0.3</td>
<td>&gt;1.7</td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.9</td>
<td>&gt;3.1</td>
</tr>
<tr>
<td>3</td>
<td>&lt;1.5</td>
<td>&gt;4.5</td>
</tr>
<tr>
<td>4</td>
<td>&lt;2.1</td>
<td>&gt;5.9</td>
</tr>
<tr>
<td>5</td>
<td>&lt;2.8</td>
<td>&gt;7.2</td>
</tr>
<tr>
<td>6</td>
<td>&lt;3.5</td>
<td>&gt;8.8</td>
</tr>
<tr>
<td>7</td>
<td>&lt;4.2</td>
<td>&gt;9.8</td>
</tr>
<tr>
<td>8</td>
<td>&lt;4.9</td>
<td>&gt;11.1</td>
</tr>
<tr>
<td>9</td>
<td>&lt;5.6</td>
<td>&gt;12.4</td>
</tr>
<tr>
<td>10</td>
<td>&lt;6.3</td>
<td>&gt;13.7</td>
</tr>
<tr>
<td>11</td>
<td>&lt;7.1</td>
<td>&gt;14.9</td>
</tr>
<tr>
<td>12</td>
<td>&lt;7.8</td>
<td>&gt;16.2</td>
</tr>
</tbody>
</table>

UPC, urine protein : creatinine.
Therefore, it is important to determine whether changing serial UPC measurements are clinically important. The RCV in human medicine is becoming widely used to determine biologic variance of biochemical analytes.22,23 RCV is defined as the statistically significant difference between 2 consecutive test results in an individual patient, and it is helpful to determine if increased or decreased serial values are likely to represent true changes or if they are compatible with biologic variation.19 In this study, the RCV was used to determine critical values for which measurements above and below represent statistically significant changes from the baseline UPC (Table 1), and the results provide the first quantitative guideline for assessing serial UPC values in proteinuric dogs.

Based on this study, the RCV graphs can be used to determine if a subsequent measurement is likely increased or decreased in XLHN dogs with a baseline UPC between 0.5 and 12. With the RCV graphs, an absolute change can be obtained from Figure 2, whereas a percentage change can be obtained from Figure 3, depending on the preference of the clinician and perceived ease of use. Variability of the UPC in proteinuric dogs outside the range represented in the graphs still needs to be determined.

When a serial UPC value is outside the calculated RCV based on the initial UPC measurement, it is highly likely that a true change in the UPC has occurred. However, if the UPC value is within the limits set by the RCV, serial monitoring becomes important to distinguish random fluctuation from a true, but small increase or decrease in the magnitude of proteinuria. For example, if a clinician finds that the UPC value in a patient has increased from 5 to 6, there is no evidence based on this study’s data to support that this increase is significant. However, if the UPC increases from 5 to 6, and then to 7, the increasing trend is worrisome, despite each serial value falling within the acceptable range for random day-to-day variation as compared with the initial UPC value of 5. In this case, a change in treatment or further evaluation to determine the cause for the increase is warranted.

The dogs in this study were genetically related, and during the 3-day evaluation periods they were subjected to similar exogenous influences and pre-analytical factors that can influence the UPC. It is this highly controlled environment that makes this study relatively accurate in its estimation of the day-to-day variability in dogs with XLHN. However, the UPCs of a random sample of dogs that have a variety of naturally occurring disease processes and that are cared for by different owners may demonstrate more variability in their UPC because of these extra factors incorporated into the estimated day-to-day variability. In addition, dogs with other glomerulopathies may demonstrate different random biologic day-to-day UPC variation than dogs with XLHN. Therefore, the variability of the UPC in dogs with other glomerular diseases still warrants investigation in order to determine to what extent these guidelines are applicable to other disease states.

The second purpose of this study was to determine the number of urine samples that should be averaged to obtain an accurate estimate of the UPC. Gibb and colleagues recommended averaging 5 urine samples to decrease UPC variation in humans, but they did not address whether a different number of samples is recommended for low and high UPC values.24 Based on the results of the present study, when the UPC is <4, reliability of that value is high, and therefore, the UPC needs to be measured only once to obtain a reliable value (Table 2). This finding suggests that a dog can generally be classified as normal or proteinuric at one point in time based on a single sample, with the caveat that additional measurements may be needed to confirm persistence of proteinuria. Also, serial monitoring is recommended to confirm mild proteinuria if the UPC value is near 0.5. At higher UPC values (>8), we find similar results to Gibb et al, where 4–5 measurements may need to be averaged to obtain a reliable UPC value. However, in many cases it may be impractical to take 5 separate urine samples to obtain a single UPC estimate owing to cost, time, a rapidly progressing disease process, or all three. Even averaging 2 measurements greatly increases the accuracy of the UPC estimate, and when possible, we recommend averaging measurements obtained from 2 to 3 separate urine collections or pooling 2 to 3 urine samples (within a 3-day period) when the UPC is >4.

Guidelines are currently not available for timing of sample collection with repeated UPC measurements. Many studies have found good correlation of the UPC with the total 24-hour protein excretion in urine samples collected at random; however, UPC variability was not evaluated. One study in humans found that the UPC had the lowest day-to-day variation in early morning urine samples compared with bedtime samples.25 No such studies have been performed in animals. Therefore, when multiple samples are being collected, whether to assess the true UPC value or for long-term monitoring, we recommend taking steps to minimize factors that can influence UPC variability. These steps include sampling at the same time of day and before eating or strenuous exercise, using fresh samples collected by the same technique each time, and analyzing the UPC with the same laboratory and the same instrument for all evaluations from a single patient.

The results of this study may provide practical recommendations for practitioners to use when monitoring UPC values, although variability of the UPC in dogs with other glomerular diseases has yet to be determined. When values are not deemed significantly different based on these recommendations, increasing or decreasing trends in the UPC remain important to determine disease progression, response to therapy, or both. In addition, the UPC should be used in conjunction with patient assessment via physical exam-
ination and other clinical parameters in order to best determine treatment options.

Footnotes

4. Dry-film chemistry auto-analyzer; Vitros 250, Johnson & Johnson Co, Rochester, NY
5. Intercooled Stata 9.0, Stata Corporation, College Station, TX

Acknowledgments

This work was supported by grants from the Texas A&M University Interdisciplinary Research Program, the Texas A&M University College of Veterinary Medicine Signature Program, Nestle Purina Pet Care Research, St Louis, MO, and the National Institutes of Health, Bethesda, MD. The work was performed at Texas A&M University, College Station, TX.

References