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Urinary Biomarkers of Chronic Kidney Disease in Veterinary Medicine: Where Do We Stand?

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Introduction

Chronic kidney disease (CKD) may be initiated by glomerular damage, tubulointerstitial damage, or both. Regardless of the cause, renal function correlates best with renal tubulointerstitial changes such as interstitial fibrosis, tubular degeneration and atrophy, peritubular capillary loss, and ultimately, destruction of functional nephrons.¹ Structural and functional evidence of kidney damage is prevalent in dogs and cats, approaching 50-90% in some studies.² However, while evidence of kidney damage is common, progressive (CKD) is typically recognized clinically at an advanced stage, when lesions are both severe and irreversible, in which case options for successful therapy are limited. This is in part because the most commonly used non-invasive diagnostics tests for renal function (e.g., serum creatinine, urea nitrogen and urine specific gravity) are fairly insensitive for tubulointerstitial injury, and they can be non-specific for renal disease. Detection of tubulointerstitial damage and altered function at an earlier stage would permit earlier interventions with renoprotective therapies that slow renal disease progression and therefore prolong survival. One promising area of study that may address this issue is the evaluation of urinary biomarkers, particularly urine proteins.

Proteinuria as an indicator of renal disease

Normal urine contains only a small amount of protein, which is due to a combined effort by both the glomeruli and the proximal renal tubules. The glomerular filtration barrier is composed of three major structural components: 1) the fenestrated capillary endothelium, 2) the glomerular basement membrane and 3) podocytes. This filtration barrier normally excludes most proteins that are the size of albumin or larger (> 60 kDa), referred to as intermediate (IMW) and high molecular weight (HMW) proteins. The normal function of this barrier depends on the normal structure and function of each component as well as a normal hemodynamic steady state.³ Proteins smaller than albumin, which are referred to as low molecular weight proteins (LMW), typically pass through the glomerular barrier. However, the receptors megalin and cubilin in the proximal tubules efficiently reabsorb these LMW proteins. Affinity of these receptors for each ligand varies depending on protein charge, size, and conformation, and ligands compete for binding sites on the receptors in situations of protein-overload.⁴ Since larger proteins, particularly albumin, comprise the bulk of the proteins in plasma, glomerular damage can lead to massive renal protein loss, whereas tubular damage generally results in only mild proteinuria. Of interest, abnormally filtered proteins may stimulate release of a variety of inflammatory, vasoactive, and fibrogenic mediators by the proximal tubular cells.⁵
Proteinuria of renal origin is clinically useful in assessing renal disease, as several studies have shown a greater magnitude of proteinuria to correlate with severity and progression of renal disease in dogs and cats. However, although magnitude of proteinuria is useful in detection and prognostic of renal disease, the evaluation of urinary protein patterns and specific urinary proteins has attracted substantial interest in recent years as a promising tool for non-invasive assessment of tubular function and for predicting progression of CKD better than total proteinuria.

**Urinary protein patterns**

The pattern of proteins as visualized by urine electrophoresis can be used to help determine whether glomerular and/or tubular damage is contributing to the proteinuria. Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most commonly used gel-based methods to assess glomerular versus tubular patterns of proteinuria. With this technique, tubular damage in the face of normal glomerular function will reveal a pattern of proteinuria consisting of predominantly LMW proteins. Glomerular damage occurring without concurrent tubular damage or dysfunction will reveal a pattern consisting of IMW and HMW proteins. This glomerular pattern can be either selective, with passage of only IMW proteins (predominantly albumin) or non-selective, with passage of both IMW and HMW proteins. More typically, there is damage to both the glomerular and tubular components resulting in a mixed pattern of proteinuria. In both dogs and people, electrophoretic patterns have been associated with prognosis and/or the degree of tubulointerstitial damage as assessed by renal biopsy. However, while sensitivity of the urine protein pattern for detecting glomerular and tubular damage was good, specificity was found to be relatively low in dogs.

**Specific urinary proteins**

In addition to protein patterns, specific proteins have been used to determine the localization and severity of renal damage as well as to detect decreased renal function and tubulointerstitial damage earlier than when using more conventional methods. Abnormal proteins in the urine secondary to renal dysfunction can be present due to several possible pathophysiologic mechanisms (Table 1) and include: 1) those that originate from filtered plasma and appear in the urine secondary to decreased reabsorption due to tubular injury; 2) those that are released from or produced by damaged tubular cells; and 3) those that are allowed to abnormally pass through the glomerular filtration barrier due to altered glomerular permselectivity (i.e., IMW and HMW proteins). In addition, proteins normally produced by the tubules (e.g., Tamm-Horsfall protein) may be decreased in the urine of patients with CKD.

**Proteinuria in human medicine**

*“Tubular proteins”*

Low molecular weight proteins have been used to assess the degree of tubular function and therefore tubulointerstitial damage. Urinary β2-microglobulin (B2M), α1-microglobulin (α1M), and retinol binding protein (RBP) have all been shown to provide prognostic information and to predict the clinical course of disease better than the
magnitude of proteinuria, serum creatinine concentration, and in some cases renal biopsy analysis.\textsuperscript{14-17} Other proteins evaluated include those released from injured tubular cells, such as N-acetyl-β-D-glucosaminidase (NAG). Similar to LMW proteins, NAG activity has been found to be more sensitive for renal dysfunction than serum creatinine concentration or total urinary protein loss, and in some studies, NAG was superior in predicting renal disease progression and response to therapy in patients with different glomerular diseases.\textsuperscript{18} However, it is important to note that increases in NAG activity may be due to increased lysosomal activity secondary to increased protein uptake instead of, or in addition to, tubular damage.\textsuperscript{19} Kim-1 and NGAL are additional tubular proteins whose expression is upregulated in damaged proximal renal tubular cells, and both have shown promise as markers of tubular injury in proteinuric renal disease.\textsuperscript{20,21} However, since NGAL is a LMW protein that also circulates in the plasma, its increase in proteinuric disease is likely due to a combination of both decreased tubular reabsorption as well as increased tissue expression.\textsuperscript{20}

"Glomerular proteins"

A number of studies have used the selectivity index to assess the degree of glomerular damage.\textsuperscript{3} This index uses a ratio of HMW proteins, such as IgG, and an IMW protein, such as albumin or transferrin. A higher ratio indicates that large proteins are able to pass through the glomerular filtration barrier, and it is associated with lack of remission in certain glomerular diseases as well as a worse prognosis, even when the magnitude of proteinuria provides no predictive information.\textsuperscript{3}

\section*{Proteinuria in veterinary medicine}

In veterinary medicine, specific urinary proteins have only recently been explored in CKD. In dogs, specific protein evaluation has revealed decreases in urinary excretion of Tamm-Horsfall protein, as well as increases in a variety of proteins including NAG, GGT, RBP, α1M, B2M, lysozyme, vitamin D-binding protein, transthyretin, transferrin, thromboxane B\textsubscript{2}, and IgG.\textsuperscript{13,22-29} All of these proteins are typically normalized to urine creatinine in order to account for varying degrees of urine concentration. Of these proteins, the most studied in veterinary medicine are the tubular proteins NAG and RBP. Both are increased in the urine of dogs with CKD as compared with normal dogs, and NAG activity may be increased in dogs with pyelonephritis as compared with patients with uncomplicated lower urinary tract infections.\textsuperscript{13,24-29} In one study of 10 dogs with CKD, urinary RBP correlated with the degree of azotemia, whereas the NAG index did not.\textsuperscript{24} In addition, ongoing studies in our laboratory support correlation of urinary RBP, but not NAG activity, with disease progression in dogs (unpublished observations). Thus far, only one published report has serially evaluated specific urinary proteins in dogs with progressive CKD.\textsuperscript{25} In this study, urinary RBP, B2M, vitamin D-binding protein, α1-microglobulin, transferrin, and apolipoprotein A1 all increased during disease progression, whereas albumin and IgG remained stable during mid- to late-stage disease.\textsuperscript{25}

In cats, both urinary NAG activity and RBP are increased in patients with CKD and/or hyperthyroidism, and both decreased with treatment of hyperthyroidism.\textsuperscript{30-36} However, NAG activity did not correlate with serum creatinine concentration, and it did
not serve as an independent predictor for the development of azotemia in geriatric cats or cats with hyperthyroidism, supporting a lack of prognostic value in these populations.\textsuperscript{32,33} For urinary RBP, there was good correlation with the degree of azotemia present, similar to dogs.\textsuperscript{34-36}

**Beyond proteinuria**

In addition to urinary proteins, cells and cellular components have been recent targets for biomarker discovery and monitoring of renal disease in human medicine. Urinary exosomes are membrane vesicles that originate from the endocytic pathway and are secreted by various renal cells.\textsuperscript{37} They provide a source of proteins that originate from all cells that line the urinary space. Therefore, they contain many proteins that are associated with renal disease and provide a rich source for biomarker discovery.\textsuperscript{37}

Podocytes have also been detected in urine using cell culture, mRNA analysis, and immunostaining of cytospin preparations.\textsuperscript{38} Increased loss of podocytes into the urine has been associated with several glomerular diseases, and in particular, their presence was associated with active glomerular injury whereas proteinuria persisted in the absence of active injury.\textsuperscript{38} However, reliable identification of viable podocytes remains a limiting factor in detection of podocyturia.

**Summary**

Measurement of specific urinary proteins is already becoming commonplace in human medicine to aid in the early diagnosis and monitoring of CKD. Many of these proteins also appear to be promising in dogs and cats with CKD. However, further evaluation is needed to better establish the role of these urinary proteins in veterinary patients as compared with conventional measures of renal disease, including histologic determination of tubulointerstitial damage. In addition, other urinary biomarkers, such as exosomes and podocytes, provide a potentially exciting area of future investigation in veterinary medicine.

Table 1: Partial listing of proteins that are either released into the urine from damaged renal tubular cells or that pass into the urine ultrafiltrate from plasma and are normally reabsorbed by proximal renal tubular cells.

<table>
<thead>
<tr>
<th>Released from damaged tubular cells</th>
<th>Reabsorbed by tubular cells (LMW proteins)</th>
</tr>
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<tbody>
<tr>
<td>N-acetyl-(\beta)-D-glucosaminidase (NAG)</td>
<td>Retinol binding protein (RBP)</td>
</tr>
<tr>
<td>(\gamma)-glutamyltransferase (GGT)</td>
<td>(\beta)2-microglobulin (B2M)</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP)</td>
<td>(\alpha)1-microglobulin ((\alpha)1M)</td>
</tr>
<tr>
<td>Glutathione-S-transferase (GST)</td>
<td>Lysozyme</td>
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<tr>
<td>Kidney injury molecule-1 (Kim-1)</td>
<td>Vitamin D-binding protein</td>
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<tr>
<td>Neutrophil gelatinase-associated lipocalin (NGAL)</td>
<td>NGAL</td>
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<td>Clusterin</td>
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References


