Universitat Autònoma de Barcelona

AVANCES EN NUEVOS MARCADORES DE DAÑO RENAL EN EL DIAGNÓSTICO Y TRATAMIENTO DE LA LEISHMANIOSIS CANINA

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Tesis Doctoral
Departament de Medicina i Cirugia Animals
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Los doctores *JOSEP PASTOR MILÁN, ASTA TVARIJONAVICIUTE* y *MARCO CALDIN,* Profesor Titular del Departament de Medicina i Cirugia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, Investigadora Posdoctoral de la Facultad de Veterinaria de la Universidad de Murcia y Director Sanitario de la Clinica Veterinaria Privata San Marco en Padua (Italia), respectivamente.

INFORMAN:

Que la memoria titulada **AVANCES EN NUEVOS MARCADORES DE DAÑO RENAL EN EL DIAGNÓSTICO Y TRATAMIENTO DE LA LEISHMANIOSIS CANINA**, presentada por **SAMANTHA GUERRERO CABRERA** para la obtención de grado de Doctor en Veterinaria y la Mención de Doctor Internacional por la Universitat Autònoma de Barcelona, ha sido realizada bajo nuestra dirección y, considerándola satisfactoriamente finalizada, autorizamos su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos oportunos, firmamos el presente informe en Bellaterra, a 25 de septiembre de 2017.

Firmado

Josep Pastor Milán

Asta Tvarijonaviciute

Marco Caldin

A mi madre y a mi padre

Viajar es marcharse de casa,
es dejar los amigos
es intentar volar
volar conociendo otras ramas
recorriendo caminos
es intentar cambiar.

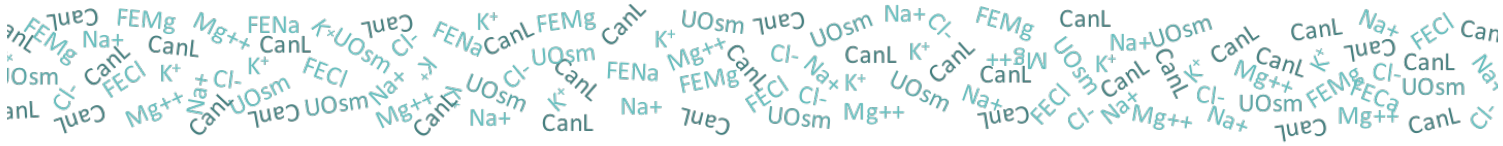
Viajar es vestirse de loco
es decir "no me importa"
es querer regresar.
Regresar valorando lo poco
saboreando una copa,
es desear empezar.

Viajar es sentirse poeta,
es escribir una carta,
es querer abrazar.
Abrazar al llegar a una puerta
añorando la calma
es dejarse besar.

Viajar es volverse mundano
es conocer otra gente
es volver a empezar.
Empezar extendiendo la mano,
aprendiendo del fuerte,
es sentir soledad.

Viajar es marcharse de casa,
es dejar los amigos
es intentar volar;
volar conociendo otras ramas
recorriendo caminos
es intentar cambiar.

Viajar es regresar *- Gabriel García Márquez*



Agradecimientos

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A mi madre por su infinita paciencia, compresión y cariño incondicional. Gracias por ser siempre mi fan número uno, y consentir todos mis proyectos. Por creer en mí, cuando ni yo misma me lo creo. Eres un ejemplo de que todo se puede realizar con un poco de fe en sí mismo.
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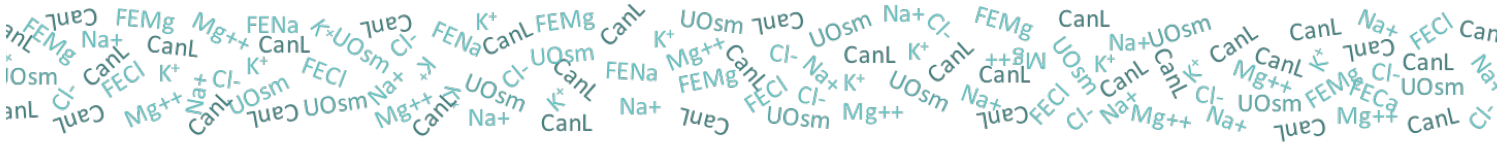
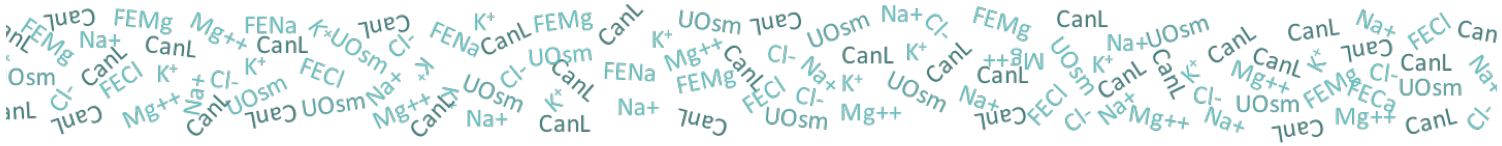


1. Índice


CanL FEMg Mg++ FENa K+ UOsm Cl- Na+ FECl Can
FEMg Na+ CanL FECl K+ Cl- UOsm Mg++ Ca

2. Resumen

La leishmaniosis canina (CanL, *canine leishmaniosis*) es una zoonosis endémica transmitida por vectores. La enfermedad renal es una de las principales complicaciones y causas de mortalidad en la CanL. En la leishmaniosis humana se ha observado disfunción renal tubular asociada con defectos en la concentración de orina y con desequilibrios electrolíticos.

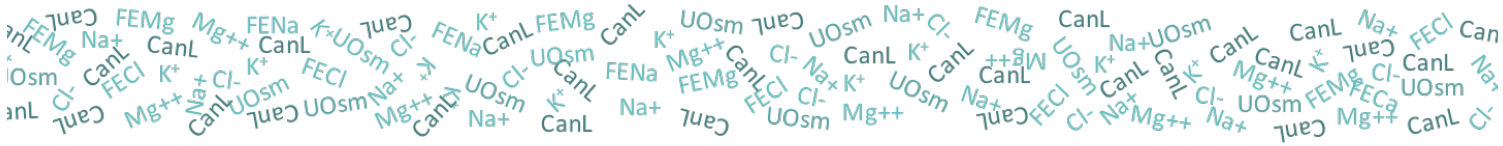
Las pruebas de laboratorio comúnmente utilizadas para evaluar la función renal carecen de una adecuada sensibilidad y especificidad, y además la progresión de la enfermedad renal es frecuentemente asintomática en las etapas iniciales de la CanL. Todo esto hace necesario encontrar nuevos biomarcadores capaces de detectar el daño renal precoz, que puedan ser útiles para diagnosticar y evaluar la progresión y la respuesta al tratamiento de la enfermedad renal secundaria a CanL.

Esta investigación se dividió en dos estudios. En el primer estudio, se realizó la validación analítica de la medición con osmometría de punto de congelación de la osmolalidad urinaria (UOsm, *urine osmolality*) en una amplia población de perros clínicamente sanos, demostrando un buen desempeño del método. Se establecieron intervalos de referencia en perros jóvenes-adultos y en perros ancianos. También se determinó el efecto de la edad, el sexo y el estado reproductivo sobre la UOsm. Los resultados mostraron que la concentración de orina disminuye en perros ancianos. No se observó ninguna influencia del sexo, pero se detectó una UOsm menor en las perras castradas en comparación con las intactas, sugiriendo un efecto de la esterilización sobre la concentración de orina.

En el segundo estudio, se evaluó la UOsm y las fracciones de excreción de Na, K, Cl y Mg en diferentes estadios de la enfermedad renal en una pequeña población de perros naturalmente infectados con *L. infantum.*

Los cambios en estos analitos se evaluaron en perros con proteinuria luego de un mes de tratamiento. Todas las fracciones de excreción evaluadas, con excepción de la fracción de excreción de K, probaron ser biomarcadores útiles para detectar disfunción tubular temprana antes de la presentación de azotemia. Sin embargo, sólo la fracción de excreción del Mg mostró una clara evidencia de la mejoría renal después del tratamiento.

Los resultados de esta investigación proveen la base para investigaciones posteriores acerca de la UOsm y las fracciones de excreción of Na, K, Cl y Mg, y su aplicación en el área clínica para el diagnóstico y monitorización del daño renal.



3. Abstract

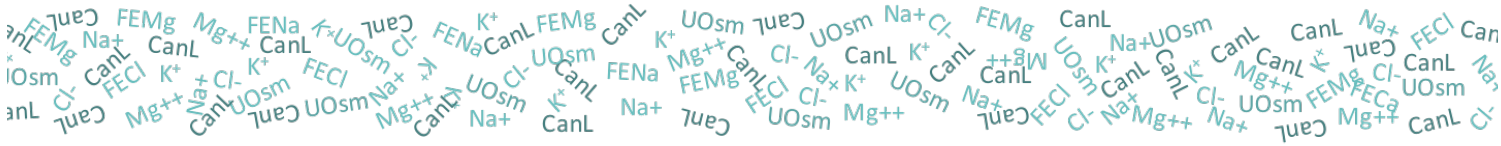
Canine leishmaniosis (CanL) is a vector-borne endemic zoonosis. Renal disease is one of the main complications and a major cause of mortality in CanL. Renal tubular dysfunction has been observed in human leishmaniosis associated with defects in urine concentration and electrolytic disorders.

Most commonly used tests for renal function assessment lack adequate sensitivity and specificity, and as renal disease progression is often asymptomatic in the initial stages of CanL, it is necessary to find new biomarkers able to detect early kidney damage, that could be useful to diagnose, evaluate the progression and the response to therapy of renal disease secondary to CanL.

This research was divided into two studies. In the first study, the analytical validation of freezing point depression measurement of canine urine osmolality (UOsm) was performed in a large population of clinically healthy dogs, showing a good performance. Reference interval were established in young-adult and senior dogs. The effect of age, sex, and reproductive status on UOsm was determined as well. The results demonstrated that urine concentration decrease in older dogs. No influence of sex was observed, but UOsm was lower in neutered than in intact female dogs suggesting an effect of sterilization on urine concentration.

In the second study, the evaluation of UOsm and fractional excretion of Na, K, Cl and Mg at different stages of renal disease was performed in a small population of dogs naturally infected with *L. infantum*. Changes of these tests after one month of treatment were evaluated in dogs with proteinuria. All evaluated fractional excretions, with exception of fractional excretion of K, proved to be useful markers to detect early tubular dysfunction before the presentation of azotemia. However, only fractional excretion of Mg showed clear evidence for renal improvement after treatment.

The results of this research provide a basis for the further investigation about UOsm and fractional excretions of Na, K, Cl and Mg, and their application in clinical settings for renal damage diagnosis and monitoring.

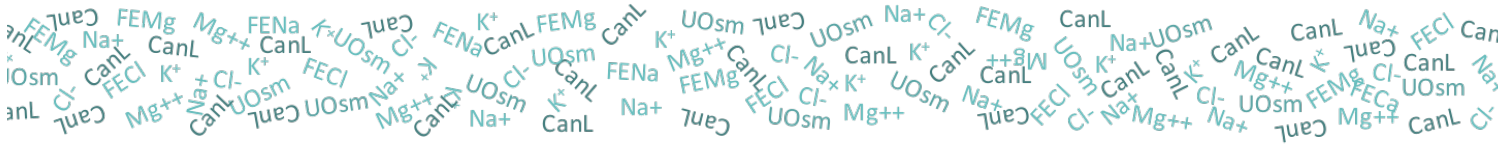


4. Abreviaturas

ACVIM	American College of Veterinary Internal Medicine
ACE	angiotensin-converting enzyme
ADH	antidiuretic hormone
AKI	acute kidney injury
ALP	alkaline phosphatase
ALT	alanine aminotransferase
APP	alanine aminopeptidase
CanL	leishmaniosis canina, *canine leishmaniosis*
CI	confidence interval
CKD	chronic kidney disease
Cl	chloride
Cr	creatinine
CV	coefficient of variation
CVs	coefficients of variation
CysC	cystatin C
FE	fracción de excreción, *fractional excretion*
FEs	fracciones de excreción, *fractional excretions*
FECl	fractional excretion of chloride
FEIgG	fractional excretion of Immunoglobulin G
FEIgM	fractional excretion of Immunoglobulin M
FEK	fractional excretion of potassium
FEMg	fractional excretion of magnesium
FENa	fractional excretion of sodium
GFR	glomerular filtration rate
GGT	gamma-glutamyltransferase
HMW	high molecular weight
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-2	interleukine-2

IL-7	interleukine-7
IL-18	interleukine-18
IRIS	International Renal Interest Society; Sociedad Internacional de Interés Renal
K	potassium
kDa	Kilodaltons
KIM-1	kidney injury molecule-1
LDH	lactate dehydrogenase
LMW	low molecular weight
LOD	limit of detection
LOQ	limit of quantification
Mg	magnesium
MHC	major histocompatibility complex
MMW	middle-molecular weigh
mOsm/kg	milliosmoles per kilogram
NAG	N-acetyl-β-D-glucosaminidase
NGAL	neutrophil gelatinase-associated lipocalin
r^2	adjusted correlation coefficient
RBP	retinol-binding protein
RIs	reference intervals
sCysC	serum cystatine C
sCr	serum creatinine
SD	standard deviation
SDMA	symmetric dimethylarginine
Se	serum electrolyte concentration
SG	specific gravity
sNGAL	serum neutrophil gelatinase-associated lipocalin
THP	Tamm–Horsfall protein
TTR	transthyretin
TXB2	thromboxane B2
uGGT	urinary gamma-glutamyltransferase

uClus	urinary clusterin
uCysC	urinary cystain C
uCPR	urinary C-reactive protein
uRBP	urinary retinol-binding protein
uCr	urinary creatinine
Ue	urinary electrolyte concentration
uFer	urinary ferritin
uIgA	urinary Immunoglobulin A
uIgG	urinary Immunoglobulin G
uIgM	urinary Immunoglobulin M
uNAG	urinary N-acetyl-β-D-glucosaminidase
uNGAL	urinary NGAL
UOsm	urine osmolality
UPC	urine protein:creatinine ratio
USG	urine specific gravity
XLHN	X-linked hereditary nephropathy



5. Introducción

La leishmaniosis canina producida por *Leishmania infantum* (sin. *Leishmania chagasi* en Latinoamérica) es una de las enfermedades protozoaria, zoonótica y trasmitida por vectores, más importantes a nivel mundial por su carácter potencialmente fatal (Solano-Gallego et al. 2011; Paltrinieri et al. 2016). La leishmaniosis canina es endémica en Europa, Asia, Norte de África y Sur América, y es una enfermedad emergente en Norte América (Duprey et al. 2006, Maia et al. 2010). En la cuenca mediterránea, se estima que entre el 65-80% de los perros han tenido contacto con *Leishmania* (Solano-Gallego et al. 2011).

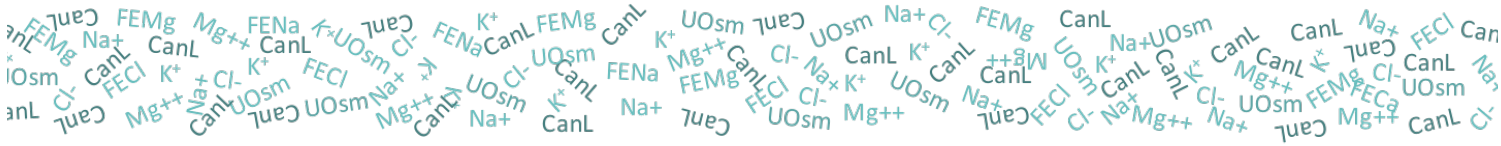
La enfermedad renal, puede ser una de las únicas manifestaciones de la leishmaniosis canina. Se caracteriza por un curso lento, progresivo y frecuentemente asintomático, siendo una de las principales causas de mortalidad de los perros con *Leishmania*. (Solano-Gallego, et al. 2011). Con menor frecuencia puede presentarse de forma aguda e hiperaguda. La disfunción renal en la leishmaniosis canina, es provocada principalmente por la respuesta inflamatoria persistente desencadenada por el depósito de inmunocomplejos a nivel glomerular y tubular causando glomerulonefritis, glomeruloesclerosis y nefritis intersticial (Paltrinieri et al. 2016). Las lesiones renales afectan la filtración glomerular y la funcionalidad tubular e intersticial, alterando la capacidad de concentrar la orina, y la reabsorción y la excreción renal de solutos como las proteínas y los electrolitos (Hokamp and Nabity, 2016).

Tanto en humanos como en perros, los métodos diagnósticos convencionales, como la creatinina y la urea séricas, y la densidad urinaria determinada por refractometría, carecen de sensibilidad y/o especificidad para identificar la enfermedad renal cuando el daño es incipiente, y poseen una menor capacidad para determinar el origen glomerular o tubular de dicho daño.

El diagnóstico precoz de la enfermedad renal en la leishmaniosis canina, podría permitir la instauración de medidas de prevención y de un tratamiento adecuado, para evitar la progresión y empeoramiento de las lesiones renales, así como para aumentar las probabilidades de supervivencia de los animales afectados.

Por dichas razones, se hace necesaria la búsqueda de nuevos marcadores biológicos, capaces de detectar un mínimo de daño renal. Recientemente se ha incluido la determinación de la dimetil arginina simétrica sérica y/o plasmática en la clasificación de la Sociedad Internacional de Interés Renal (IRIS, *International Renal Interest Association*) de la enfermedad renal crónica, demostrando la importancia y potencial de la investigación en los nuevos biomarcadores de daño renal. La detección de marcadores biológicos más sensibles y específicos de lesión a nivel glomerular o tubular, pueden convertirse en una herramienta útil para detectar estadios iniciales de daño renal, valorar la severidad de las lesiones y la recuperación de la funcionalidad, la evolución y la respuesta, al tratamiento en la enfermedad renal secundaria a la leishmaniosis canina.

En esta investigación se validó la medición de la osmolalidad urinaria por medio de la osmometría de punto de congelación, se establecieron intervalos de referencia según la edad, y se determinó el efecto de la edad, el género y el estado reproductivo, sobre la concentración urinaria en perros sanos. Posteriormente, se estudiaron los cambios en la osmolalidad urinaria y las fracciones de excreción de sodio, potasio, cloro y magnesio en diferentes estadios de enfermedad renal en perros naturalmente infectados con *Leishmania*, y la dinámica de dichos analitos en el seguimiento de la evolución de la enfermedad y la respuesta al tratamiento.



6. Objetivos

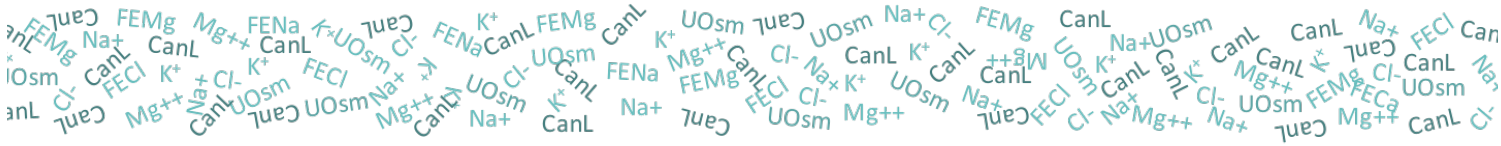
1. Realizar la validación analítica de la osmometría de punto de congelación como método para medir la osmolalidad urinaria en perros sanos.

2. Establecer intervalos de referencia de la osmolalidad urinaria según la edad en perros jóvenes-adultos, y en perros ancianos.

3. Evaluar los efectos de la edad, sexo, y el estado reproductivo en los valores de la osmolalidad urinaria en perros sanos.

4. Determinar los cambios de la osmolalidad urinaria, y las fracciones de excreción de sodio, potasio, cloro y magnesio, en diferentes estadios de la enfermedad renal secundaria a la leishmaniosis canina.

5. Investigar los cambios en los valores de la osmolalidad urinaria y las fracciones de excreción de sodio, potasio, cloro y magnesio, al momento del diagnóstico y después de un mes de tratamiento en diferentes estadios de la enfermedad renal secundaria a la leishmaniosis canina.



7. Revisión Bibliográfica

7.1 Renal disease in canine leishmaniosis

Nephropathy in CanL is a multifactorial disorder that undergoes chronic evolution. It has been proposed that renal disease in CanL is mainly produced due to high serum concentration of immune complexes and its tissue deposition, vasculitis secondary to activation of the complement system and antihistone antibodies presented in circulation within the glomeruli causing glomerulonephritis (Torrent et al. 2005; Koutinas and Koutinas, 2014).

However, one study by Costa et al. (2010) proposed that immunoglobulins and complement play no role in the pathogenesis of advance stages of glomerulonephritis. As an alternative, these authors suggested that perpetuation and progression of the underlying glomerular disease in CanL was associated to hypercellularity secondary to migration of inflammatory cells and/or decreased apoptosis. The presence of *Leishmania* antigen in mesangial cells that probably guides inflammatory infiltrate of $CDT^{+}4$ cells, and the decrease in apoptosis mediated by low production of tumoral necrosis factor, both contributes to hypercelullarity. Adhesion molecules as P-selectin and intercellular adhesion molecule-1 that migrate with inflammatory cells help to determine the proliferative pattern of glomerulonephritis (Costa et al. 2010).

Recently, severity of the glomerular lesion in CanL has been related to an increase in the inflammasone complex characterized by the increase in glomerular nucleotide-binding domain leucine-rich repeat-containing-like receptor family, pyrin domain containing 3 and autophagosome-associated microtubule-associated protein 1 light chain 3 (Esch et al. 2015). Inflammasome activity helps in host defense by activation of a rapid inflammatory response and restriction of pathogen replication, but also contributes to the development and progression of chronic

pathologies such as renal disease due to *Leishmania* infection (de Zoete et al. 2014).

Renal disease in CanL can be presented in the forms of acute kidney injury (AKI) to chronic kidney disease (CKD) and be asymptomatic or symptomatic. The asymptomatic presentation often course with proteinuria. Progressive proteinuria could lead to tubular excretory dysfunction, reduction or increment of glomerular filtration rate (GFR) and systemic hypertension. Intense proteinuria could cause muscular wasting and cachexia, and occasionally pulmonary thromboembolism. Severe proteinuria and end stage kidney disease are the principal cause of death in chronic *Leishmania* cases in dogs (Koutinas and Koutinas, 2014).

Renal tubular dysfunction in *Leishmania* infection has been observed in asymptomatic humans. Its seems to be related to urinary concentration and/or acidification deficits, presented before a decrease GFR and an increase in serum creatinine (sCr) (Silva Junior et al. 2014). Urinary concentration deficit has been associated with lower expression of aquaporine 2 and an increase in the expression of cotransporter Na-K-2Cl as compensatory mechanism in renal tubules producing lower urine osmolality (UOsm). Acidification deficit has been related to abnormalities in the renal transporters implied in acid-base regulation including an increased expression of Na/H exchanger 3 in the proximal tubule, and H-ATPase and pendrin in the distal tubule. Renal tubular dysfunction has also been related to serum and urinary electrolytic disturbances in humans (Lima Verde et al. 2007; Oliveira et al. 2011; Silva Junior et al. 2014).

7.1.1. Histopathology

Histopathological studies about CanL demonstrate glomerular lesions in all animals involved (Costa et al. 2003; Zatelli et al. 2003). Chronic

glomerulonephritis represents the outcome of several types of glomerulonephritis probably because of the long evolution of the disease in dogs, even when the parasite was probably eliminated from the tissue (Costa et al. 2003).

Glomerular lesions in CanL have been histologically classified as minor glomerular abnormalities, focal segmental glomeruloesclerosis, proliferative glomerulonephritis, membranous glomerulonephritis, membranoproliferative glomerulonephritis, crescentic glomerulonephritis and chronic glomerulonephritis (Costa et al. 2003; Zatelli et al. 2003).

Glomerulonephritis and diffuse mesangial proliferative glomerulonephritis are the most frequent patterns observed in CanL in Brazil and Italy (Costa et al. 2003; Zatelli et al. 2003). In Spain (Nieto et al. 1992) and France (Benderitter et al. 1988), diffuse membranoproliferative glomerulonephritis was the most frequent histopathologic pattern observed in naturally infected dogs (Costa et al, 2003). The variation presented between countries could be related to the number of animals in the studies and different species of *Leishmania* involved in each country (Hosein et al. 2017).

Focal segmental glomerulosclerosis and ultrastructural glomerular changes in normal histology samples have been found in leishmaniotic dogs, non proteinuric to borderline proteinuric, with normal plasma Cr (Costa et al. 2003).

Costa et al. (2003) observed tubular changes in 96% (53 of 55) and interstitial nephritis in 78% (43 of 55) of the animals studied. Zatelli et al. (2003) observed tubulointerstitial histologic changes less frequently. Inflammation affected principally the renal cortex and was characterized by organized foci with lymphocytes, plasma cells, and scarce histiocytes and polymorphonuclear neutrophils. Diffuse inflammatory infiltration

was severe, less frequent and was characterized by small clusters to massive foci and intertubular cords spreading in areas of fibrosis. Renal cortex was the most affected by interstitial nephritis. In the Costa et al. study, even in the cases when there was inflammatory infiltrate in the medulla (26%), also the lesions were more severe in the cortex. The most frequent tubular change was vacuolar and hyaline degeneration, also presented without interstitial inflammatory infiltration and atrophy. It has been observed that isolated tubules with severe inflammatory foci develop necrosis. These changes are potential causes of renal dysfunction in CanL (Costa et al. 2003).

It has been proposed that tubulointersticial histopathologic changes are secondary to glomerular pathology, immune complex mediated inflammation and renal interstitium fibrosis (Koutinas and Koutinas, 2014), rather than a primary lesion due to *Leishmania* infection.

7.2 Urinary renal biomarkers in CanL

Biological markers known as biomarkers, are valuable tools to identify normal or pathogenic processes, or the response to treatment, and are helpful to determine patient status. Biomarkers are often used to diagnose and measure a pathological condition and prognosis of the disease. They are not necessarily involved or play a role causing the disease process (Tesch et al. 2010).

To be useful in the clinical setting, an ideal renal biomarker should be measured easily, non-invasively, accurately and reproducibly. Renal biomarkers could localize kidney injury (i.e., glomerular level, tubular level or both), sensitively indicate renal injury and predict its severity, differentiate renal injury from pre-and post-renal causes, identify or differentiate specific types of renal injury or kidney disease, diagnose

non renal injury, and monitor the kidney response to treatment. Renal biomarkers should provide useful and cost-effective clinical information that is easy to interpret, gives additional information to conventional clinical parameters, and it is applicable across a variety of populations (Tesch et al. 2010; De Loor et al. 2013).

Urine is one of the most promising biological fluids to research for earliest biomarkers of kidney injury because is easy to obtain and is closely related to the kidney. Renal biomarkers in urine could be determined by the evaluation of specific proteins or proteome profile analysis that contributes with a global overview of proteins and peptides in urine (De Loor et al. 2013). Changes in renal handling of water and other particles (urine osmololality) and variation of urine excretion of solutes such as electrolytes (fractional excretions) are also useful as renal biomarkers.

7.2.1. Markers of glomerular damage or dysfunction

7.2.1.1 Glomerular filtration rate (GFR)

Glomerular filtration rate is defined as the volume of ultrafiltrate produce per unit of time by glomerular filtration. GFR is considered one of the best methods to evaluate kidney function (Brown and Lefebvre, 2008). GFR could be affected by several extra-renal factors including sex, age, breed, weight, dietary protein intake, hydration status, sodium balance, exercise, and day-to-day circadian rhythm. The most commonly used GFR standardization method is the body weight in kg and the body corporal surface (Brown and Lefebvre, 2008; Von Hendy-Willson and Pressler, 2011).

Direct determination of GFR has been performed using the clearance of molecules freely filtered by the glomerulus with minor or no secretion and tubular reabsorption, and is not bound to plasma proteins (Brown and Lefebvre, 2008). Indirect estimation of GFR is commonly done in the clinical setting by the measurement and interpretation of serum biomarkers as Cr (Von Hendy-Willson and Pressler, 2011).

The standard method to measure GFR is renal clearance of inulin. The filtered load is equal to the total amount of this marker that undergo urinary elimination over a period. Nonetheless, this method is impractical and time-consuming because to perform the measurement is necessary the maintenance of a constant plasma concentration by constant infusion of the marker, the accurate determination of total urine volume using metabolic cages or indwelling catheters, and precision and accuracy to measure the marker (Brown and Lefebvre, 2008). Plasma clearance methods have also been used to measure GFR by determining the reduction in plasma concentration of a marker over time. The plasma concentration-versus-time curve is determined by obtaining multiple plasma samples at set time intervals for a predetermined length of time (Brown and Lefebvre, 2008). Clearance of molecules such as Cr, iohexol and radiolabeled markers have also been used to determine GFR in dogs. Other techniques include the application of contrast-enhanced computed tomography to indirectly measure an injected marker by determining it renal uptake (Von Hendy-Willson and Pressler, 2011). However, lack of standardization of protocols and complications in the reproducibility of results make the GFR measurement in dogs difficult to apply in the clinical setting (Von Hendy-Willson and Pressler, 2011).

GFR is commonly decreased by the reduction of renal artery pressure secondary to hypotension or effective circulating volume depletion (Von Hendy-Willson and Pressler, 2011). Renal mass loss of 5/6 has been

related to a 65% decrease in GFR, experimentally in dogs. AKI is generally associated with an acute decrease of GFR, and CKD with it progressive decline (Brown and Lefebvre, 2008).

One study evaluated the GFR changes in dogs naturally infected with *Leishmania*, showing that most of the proteinuric non azotemic or mildly azotemic dogs had low GFR, but glomerular hyperfiltration occurs in few dogs. Glomerular hyperfiltration was probably due to early kidney injury and/or the result of systemic hypertension. In consequence, GFR measurement could be helpful to detect early renal damage in CanL (Cortadellas et al. 2008).

7.2.1.2. Serum Creatinine (sCr)

Creatinine is a heterocyclic, nitrogenous compound with a low molecular weight (113 kDa) produced as the result of normal muscle metabolism (Relford et al. 2016). It is originated by spontaneous, irreversible, nonenzymatic, internal dehydration of creatine and dephosphorylation of phosphocreatine. Creatine and Cr are originated from endogen glycine, arginine, and methionine. A relatively minor source of Cr is ingested during consumption of animal tissue and absorbed from the intestines. Daily and almost constant production of Cr occurs in the kidney, and accounts for 2% of the total body pool of creatine (Braun et al. 2003). In dogs, endogenous Cr production accounts for about 90% of sCr (Concordet et al. 2008).

Under normal conditions, plasma Cr is filtered freely through the glomerulus and is not reabsorbed in the tubules. Consequently, its serum concentration is the same to the glomerular filtrate. However, in the proximal renal tubules Cr is weakly secreted, being most prominent in males but without significance even in male dogs with CKD (Braun et

al. 2003).

Urinary elimination of Cr is constant over time. Urinary creatinine (uCr) values do not have daily variations, but could increase or remain stable after meals. Inter-individual variation of 24 hours uCr has been observed probably due the diverse protein percentage in dietary intake, accuracy of the measurements and changes in urine dilution/concentration (Braun et al. 2003).

Serum creatinine is influenced by age being significantly lower in puppies than in adult dogs (Rørtveit et al. 2015), and is higher in large breeds (Braun and Lefebvre, 2008). Weight and/or muscle mass is the most probable reason in both cases (Concordet et al. 2008). No effect of gender has been reported in creatinine urinary elimination (Hokamp and Nabity, 2016) but moderately higher plasmatic concentrations has been observed in male dogs (Braun and Lefebvre, 2008). Also, sCr concentration could by influenced by hydration state, physical effort, secondary diminution of GFR by nephrotoxic drugs, alter renal hemodynamics by drugs and extracellular dehydration (Braun et al 2003).

Serum creatinine (sCr) values increases above the reference interval with at least 75% of nephron mass loss. Studies in experimental partially nephrectomized dogs demonstrate that this percentage corresponds to 35-60% of decrease in renal function. The difference between mass loss and renal function is in part due to compensatory mechanisms as renal hypertrophy (Hokamp and Nabity, 2016).

In practice, sCr concentration is commonly used as an indirect marker of GFR in dogs because it increases exponentially as GFR declines (Relford et al. 2016). Increment in sCr has been demonstrated in CKD and AKI, but its concentration is not useful to differentiate between them. However, sCr is one of the principal markers used by the IRIS

guidelines to classify AKI and CKD, and determine the presence and the severity of the azotemia in dogs (Table 1). Even sCr concentration increases according to the progression of CKD, it has a marked inter-individual variation, being a poor predictor of changes in GFR in some dogs (Brown et al. 2003). Has been reported that sCr could be more sensitive for detecting decreased renal function than serum urea nitrogen (Hokamp and Nabity, 2016) but is less sensitive than other renal markers to detect early renal damage (Paltrinieri et al. 2016). It seems that sCr could be a better biomarker for monitoring changes when the renal disease is established or to assess the efficiency of the treatment (Braun et al. 2003; Ghys et al. 2014).

Table 1. Staging of CKD based on blood creatinine concentration*.

Stage	Blood creatinine	Comments
At risk	<1.4	At risk
1	<1.4	Non azotemic. Other renal abnormalities such as inadequate urinary concentration without non renal cause or proteinuria of renal origin
2	1.4-2.0	Mild renal azotemia. Clinical signs mild or absent
3	2.1-5.0	Moderate renal azotemia. Many extrarenal clinical signs may be presented
4	<5.0	Increasing risk of systemic clinical signs and uremic crises

*Adapted from Elliot J, Watson AD. Chronic kidney disease: International Renal Interest Society staging and management. In: Bonagura JD, ed. Twedt DC. Kirk's current veterinary therapy. 15th ed. Chapter 189. United States of America, Missouri: Saunders, 2014:857-863.

Moreover, sCr evaluation is used to classify the severity of CanL by the LeishVet guidelines, according to the IRIS staging of CKD mentioned above (Solano-Gallego et al. 2011).

In renal disease due to *Leishmania* infection, significant negative

correlation between sCr and GFR has been observed in non azotemic and proteinuric dogs. However, some of these dogs had high GFR indicating glomerular hyperfiltration, that could be observed in early glomerular damage and predicts nephropathy development. This discrepancy shows that sCr could mislead changes in GFR and its lack of sensibility to detect early renal damage in non azotemic, proteinuric dogs (Cortadellas et al. 2008). Also, has been reported that sCr values showed no changes associated with improvement in proteinuria after one month of treatment, in non azotemic proteinuric dogs with renal disease due to leishmaniosis (Pardo-Marín et al. 2017).

7.2.1.3. Serum urea nitrogen

Urea is a nitrogenus end-product, with a molecular mass of 60.06 g/mol, water-soluble and neutral charged molecule. Biosynthesis of urea occurs mainly in the liver from ammonia generated by catabolism of dietary proteins in the intestines, or from endogenous tissue proteins. Ammonia originated by the colon flora from protein that escapes absorption in the small bowel and by recycled urea also contribute to the urea cycle passing into the liver through the portal circulation (Wang et al. 2014).

Urea biosynthesis could be regulated by hormones such as insulin and glucagon, growth hormone and insulin growth factor-1, and could be affected by endogens and exogenous glucocorticoids. Blood urea values are also affected by tissue breakdown, high protein intake, and major gastrointestinal hemorrhage (Wang et al. 2014).

Serum/plasma urea nitrogen values are decreased until 50% in dogs between birth and 1-2 months. No gender effect has been reported. Lipemia, hemolysis and ictericia interfere with urea nitrogen measurements (Braun and Lefebvre, 2008).

About 90% of urea is eliminated in urine mostly by glomerular filtration and in lower proportion by tubular secretion in the thin segment of Henle (Hosten, 1990; Wang et al. 2014). Almost half of the urea is reabsorbed in the proximal tubules. The amount of urea resorbed in the collecting ducts is dependent on the permeability and the tubular concentration of urea in this segment. Both factors are affected by the presence of antidiuretic hormone (ADH). The reabsorption of urea in the medullary collecting ducts and its intrarrenal recycling contribute to create the osmotic gradient, critical concentrating urine (Hosten, 1990). A small amount of urea is lost through the gastrointestinal tract, lungs and sweat (Wang et al. 2014).

In the normal kidney, at high flow rates, approximately 40% of filtered urea is reabsorbed. At low flow rates, approximately 60% of filtered urea is reabsorbed and added back to the blood urea concentration. This explains the high urea nitrogen levels seen when GFR decrease by any cause. Increment in urea nitrogen could be secondary to pre-renal, renal and post-renal causes, and due to increased catabolism and/or protein digestion. Decreased urea nitrogen values could be the result of decreased protein intake or protein anabolism, increased excretion as in any case of polyuria and decrease in production as in liver failure (Braun and Lefebvre, 2008).

Serum/plasma urea nitrogen is considered a screening test of renal function when is interpreted together to sCr values. However, urea measurement is less specific than sCr for the diagnosis and management of CKD (Braun and Lefebvre, 2008) and it is influenced by dehydration, gastrointestinal hemorrhage or increase in protein catabolism.

7.2.1.4. Urine protein: creatinine ratio (UPC)

The glomerular filtration barrier is the main mechanism for preventing proteinuria. This barrier allows free filtration of proteins <40 kDa (LMW, *low molecular weight*) to filter freely. Middle molecular weight (MMW) proteins are largely restricted, and high molecular weight (HMW) proteins (>100 kDa) are almost completely restricted from glomerular filtration. As well, the proximal tubular epithelial cells are responsible to reabsorb proteins normally present in the urinary filtrate, to prevent proteinuria (D'Amico and Bazzi, 2003).

Proteinuria is consequence of the impairment in the glomerular barrier permeability that allows filtration of albumin and HMW proteins. Abnormally filtered proteins increased the load allowed by the renal tubules and saturate the reabsorption mechanism. This situation induces toxic damage in the proximal tubules leading to an insufficient protein reabsorption, mainly of the LMW iproteins (D'Amico and Bazzi, 2003). The detection of increase in protein excretion is useful to the diagnosis and prognosis in the early detection and confirmation of renal disease. Its quantification could be useful to assess the effectiveness of therapy and the progression of the disease (Price et al. 2005).

The urine protein:creatine ratio (UPC) is a measurement that allows to quantify proteinuria (Paltrinieri et al. 2016) in spot samples, based on the assumption that excretion of Cr and protein is almost constant throughout the day when the GFR is stable (Price et al. 2005). The value of UPC could be affected by contamination from the low urinary tract during collection, and by the presence of active urinary sediment (Paltrinieri et al. 2016). In dogs, long-term glucocorticoid therapy produced a regular increase of UPC from 0.5 at 2 weeks, to above 1 after 4 weeks (Braun and Lefebvre, 2008).

IRIS guidelines (Table 2) and the American College of Veterinary Internal Medicine (ACVIM) proteinuria consensus statement, recommend cut-off points or decision limits to evaluate proteinuria in dogs with renal disease (Littman et al. 2013).

Table 2. Substaging of CKD based on urine protein:creatinine values*

UPC values	Substaging
>0.2	Non proteinuric
0.2-0.5	Borderline proteinuric
>0.5	Proteinuric

*From Elliot J, Watson AD. Chronic kidney disease: International Renal Interest Society staging and management. In: Bonagura JD, ed. Twedt DC. Kirk's current veterinary therapy. 15th ed. Chapter 189. United States of America, Missouri: Saunders, 2014:857-863.

UPC provides an indication of altered glomerular permselectivity, as has been shown in X-linked hereditary nephropathy (XLHN), a model of glomerular disease in dogs (Nabity et al. 2012). UPC values ≥2 could be indicative of glomerular proteinuria, while values <2 are often present in tubular proteinuria. Nonetheless, it has been observed that some proteinuric dogs with UPC <2 had primary glomerular damage confirmed by renal biopsy. Consequently, UPC values could not always differentiate glomerular from tubular damage (Cianciolo et al. 2016). Recently, has been reported method-dependent analytic variability (pyrogallol red molybdate versus Coomassie brilliant blue) in the measurement of total proteinuria that could affect the final interpretation of the UPC ratio and reduce the ability to correctly classify dogs with UPC < 0.2 (Rossi et al. 2016).

Otherwise, persistent renal proteinuria quantify by UPC levels, could be an early indicator and a negative prognostic factor of CKD in dogs

(Hokamp et al. 2016). High UPC values have been considered a risk factor for the progression of nephropathy (Paltrinieri et al. 2016) and a risk factor of mortality in AKI (Brown et al. 2015).

Proteinuria should be assessed in any dog suspected or diagnosed with *Leishmania* infection, according to the ACVIM consensus for glomerular diseases (Littman et al. 2013). Usually, urine dipstick is a good screening test to first assess proteinuria, but UPC is preferred to diagnose and follow-up CanL. UPC values are used to classify the severity of the disease by LeishVet guidelines (Solano-Gallego et al. 2011), according to proteinuria classification by IRIS guidelines. Proteinuria also has been proposed as a negative prognostic factor in dogs with leishmaniosis (Paltrinieri et al. 2016)

One study in CanL at different stages of renal disease reported a significant correlation between UPC and measured GFR values. However, it seems that only a small proportion of the variability in GFR can be predicted from changes in UPC (Cortadellas et a. 2008).

Frequently, UPC values are used to monitor the treatment in CanL. One study demonstrates the reduction in the magnitude of proteinuria at diagnosis, after one month of treatment in sick dogs [stage C] (Pierantozzi et al. 2013).

7.2.1.5. Symmetric dimethylarginine (SDMA)

Symmetric dimethylarginine (SDMA) is a low molecular methylarginine (202 g/mol), positive charged and produced by obligate post-translational modification and methylation of arginine residues of various proteins in the nucleus of all cells. Free methylarginines are released into the cytosol after proteolysis and pass to the bloodstream.

SDMA has an integral role in basic cellular metabolism and is mainly excreted by glomerular filtration (90%) (Relford et al. 2016; Hall et al. 2016).

In dogs, SDMA is not affected by breed, gender or by muscular mass like sCr (Relford et al. 2016; Dahem et al. 2017). SDMA increases by age as GFG decreases, when renal function declines. Hemoglobin, lipids, bilirubin, arginine, monomethylarginine, asymmetric dimethylarginine, and homocitrulline did not interfere with SDMA measurement in humans and dogs (Relford et al. 2016).

SDMA values did not change with acute inflammatory response, hepatic disease, cardiovascular disease or diabetes without concurrent renal disease in humans (Relford et al. 2016). In dogs, there was no correlation between liver enzymes (ALT, ALP, GGT) and cardiac biomarkers (N-terminal pro–brain natriuretic peptide) (Relford et al. 2016).

SDMA has been proposed as a renal biomarker of glomerular damage because its concentration is highly and inversely correlated with GFR (Dahlem et al. 2017). In XLHN has been demonstrated that, SDMA consistently detected <30% loss of renal function using either a general reference interval or serial measurements, being useful to diagnose and monitor the renal disease (Nabity et al. 2015).

SDMA accurately and precisely estimated GFR, being more sensitive than sCR in humans and dogs (Relford et al. 2016). Higher SDMA concentrations has been observed in dogs with renal azotemia compared to healthy dogs. However, no difference between SDMA values in AKI and CKD dogs was shown. The correlation between SDMA and sCr values in AKI is lower than in dogs with CKD, probably because the greater effect of muscular mass loss on sCr in CKD. Also, SDMA/Cr was higher in dogs with CKD compared to AKI ones. A SDMA/Cr ratio

>10 has been reported to give a poor prognosis in CKD (Dahlem et al. 2017).

SDMA is a more sensitive marker of reduced renal function than sCr in CKD (Fleck et al. 2003) being useful to detect early stages of CKD without increase in sCr in humans and dogs (Relford et al. 2016). Trending of sCr and SDMA could be a useful tool in the absence of baseline values, to identify early changes in renal function. However, it seems that proteinuria appears first than changes in SDMA concentrations in the presence of renal dysfunction (Nabity et al. 2015).

Recently, serum SDMA has been evaluated in CanL with renal disease. It has been reported that SDMA levels did not change when renal proteinuria improves after 1 moth of treatment. In these cases, UPC instead of SDMA would be the recommended test to monitor the therapy (Pardo-Marín et al. 2017).

7.2.1.6. Immunoglobulins

Immunoglobulins are involved in host defense mediated by antibodies. They are high weight glycoproteins produced by plasma cells in bone marrow, lymph nodes and spleen. The monomeric form of immunoglobulin M (IgM), G (IgG) and A (IgA) weight 900 kDa, 150 kDa and 160 kDa each one. IgA has a dimeric and a polymeric form (Hokamp and Nabity, 2016).

These immunoglobulins cannot pass through the normal glomerular filtration barrier because of their weight. When glomerular injury occurs, they initiate to appear into the urinary filtrate (Hokamp and Nabity, 2016). Urinary IgG (uIgG) concentration has been reported unaltered by hematuria, hemoglobinuria, pyuria and/or urinary tract infection in dogs (Hokamp and Nabity, 2016).

Increase in serum IgG and IgA on Western blot and uIgG:creatine ratio (uIgG/Cr) has been observed in AKI secondary to babesiosis (*Babesia rossi)* and leptospirosis. High levels of uIgG/Cr correlated positively to proteinuria, have been reported in dogs with pyometra associated to glomerular damage. Instead, low values of urinary IgA (uIgA) has been observed in those dogs. There have also been high levels of uIgG/Cr suggesting glomerular dysfunction in dogs with hyperadrenocorticism and snake envenomation (Hokamp and Nabity, 2016).

High concentration of uIgG and uIgM have been shown in dogs with CKD (Hokamp et al. 2016). uIgG/Cr progressively increased in CKD secondary to XLHN, compared to healthy dogs of the same age. Also, uIgG/Cr had tendency to increase before UPC in this disease, contrary to other nephropathies where uIgG/Cr appearance is observed at the same time of proteinuria. IgG has a strong correlation with UPC values showing its ability to indicate a dysfunction in glomerular permeability (Nabity et al. 2012). uIgG/Cr has been also positive correlated to histopathological findings of glomerular and tubulointerstitial damage in dogs with XLHN (Nabity et al. 2012).

Likewise, increments in uIgG/Cr and uIgM/Cr has been positively correlated to immune complex-mediated glomerulonephritis (Nabity et al. 2012, Hokampt et al. 2016). Also, increases in uIgM/Cr concentrations provided the best indication of ultrastructural glomerular damage compared to other biomarkers of renal disease, and it was related to increase risk of renal failure and death in both humans and dogs (Hokamp et al. 2016). Lower uIgM/c has been observed in juvenile nephropathies, non-immune complex-mediated glomerulonephropathies, and primary tubular disease (Hokamp et al. 2016).

In addition, fractional excretion of IgM (FEIgM) and IgG (FEIgG) has

been evaluated in proteinuric CKD dogs, showing that fractional excretion of immunoglobulins was better than its urine concentration to assess tubular damage. FEIgG has shown a strong correlation with histological tubular dysfunction, while FEIgM was equally correlated to histological glomerular and tubular damage (Hokamp et al. 2016).

On the other hand, uIgG (Solano-Gallego et al. 2003) and uIgA (Solano-Gallego et al. 2003; Zaragoza et al. 2003; Todolí et al. 2009) have been found in proteinuric dogs with *Leishmania* infection. The presence of urine antibodies in CanL is mainly due to free filtration of immunoglobulins secondary to glomerular damage (Solano-Gallego et al. 2003). In the case of IgA, has been proposed that a small proportion corresponds to local production associated with tubulointerstitial nephritis or lesions in other urinary or genital organs (bladder, urethra and/or prostate) (Todolí et al. 2009).

Lower concentrations of uIgA than uIgG have been found in CanL, even in the presence of severe glomerular damage, probably because of lower IgA than IgG serum levels (Zaragoza et al. 2003). There has been reported a lower correlation of uIgA to proteinuria, sCr and serum urea in comparison to uIgG correlation, that shows less sensitivity of uIgA to evaluate renal damage in CanL (Todolí et al. 2009).

Recently, it has been reported that uIgG/Cr increase according to the development of renal disease (Pardo-Marín et al. 2017), and that is positive correlated to proteinuria progression in CanL (Solano-Gallego et al. 2003; Pardo-Marín et al. 2017). Also, uIgG/Cr decrease has been associated to improvement of proteinuria after one month of treatment (Pardo-Marín et al. 2017) in cases without changes in baseline sCr and serum SDMA. The magnitude of its decrease was higher than other biomarkers of glomerular damage such as uCPR/Cr (Pardo-Marín et al. 2017).

7.2.1.7. Cystatin C (CysC)

Cystatin C (CysC) is a non-glycosylated protein from the superfamily of cysteine protease inhibitors, positive charged and with a LMW (13kDa). It is produced by all nucleated cells at a constant rate and released during phagocytosis acting as a proteinase inhibitor to mediate inflammation (García-Martínez et. al 2015; Hokamp and Nabity, 2016). CysC is freely filtered in glomeruli and completely absorbed and catabolized by the proximal tubular cells without tubular reabsorption or secretion (Ostermann and Joannidis, 2016). However, it has been reported that CysC can be found in small quantities in the urine with normal renal function in humans (Ghys et al. 2014). An increase in CysC has been specifically related with proximal renal tubular damage (Hokamp and Nabity, 2016).

Some extra-renal factors have been suspected to alter the production rate of serum CysC (sCysC), such as systemic inflammation, thyroid dysfunction, glucocorticoid disorders, corticosteroid therapy and HIV disease in humans (Ghys et al. 2014; Ostermann and Joannidis, 2016). As well, it values could be affected by hyperbilirubinemia and hypertriglyceridemia (Ostermann and Joannidis, 2016). However, significantly higher CysC has been observed in dogs with CKD compared to dogs with non renal diseases like immune-mediated, endocrine, dermatologic, cardiologic and neoplastic disorders (Ghys et al. 2014).

High levels of CysC were related with malignancies like melanoma and colorectal neoplasia in humans. Instead, seems CysC is not influenced by inflammation or neoplasia in dogs (Wehner et al. 2008; García-Martínez et al. 2015).

Serum CysC values could identify the presence of AKI one or two days

earlier than sCr concentration in humans with ≥2 predisposing factors of AKI. In dogs, the rol of sCysC in AKI is controversial, and it seems it could not be a sensitive indicator of decrease GFR, but higher values have been observed in severely ill dogs with hypovolemic-shock compared to healthy dogs probably related to decrease GFR (Ghys et al. 2014).

In humans and dogs, CysC is better than sCr to detect renal dysfunction related to decreased GFR in CKD. Also, in human CKD allows a better mathematical estimation of GFR (Ghys et al.2014). In dogs, it has been reported that sCysC has better sensitivity and higher negative predictive value compared to sCr to detect a diminution in GFR related to early kidney injury (Wehner et al. 2008).

Urine cystatin C (uCysC) values are much lower in healthy humans than in individuals with renal tubular damage. Also, higher uCysC levels could be useful to differentiate cases of proteinuria secondary to proximal tubular damage from cases of proteinuria without renal tubular injury in humans (Ghys et al. 2014). However, massive proteinuria could inhibit the tubular reabsorption of CysC, leading to increase in uCysC that could underestimate tubular function. In consequence, it is important to evaluate total proteinuria when uCysC is measured (Ghys et al. 2014). Also, uCysC values could be useful to predict renal replacement requirements in patients with non-oliguric ATN, but could not differentiate between AKI and CKD in humans (Ghys et al. 2014). In dogs, uCysC and urine cystatin C:creatinine ratio (uCysC/Cr) are useful to differentiate between animals without renal disease and dogs with renal disease and azotemia (Monti et al. 2012).

Urine cystatin C values tend to increase according to severity of CKD in dogs with *Leishmania*, but the increase was only significant in proteinuric and azotemic dogs, suggesting that uCysC would not be

useful to identify early kidney dysfunction in CanL (García-Martínez et al. 2015).

7.2.2. Markers of tubular damage or dysfunction

7.2.2.1. γ-glutamyl transferase (GGT)

GGT is an enzyme of the luminal side of the brush border of the proximal renal tubular cells essential in glutathione homeostasis being implied in the extracellular glutathione breakdown and as a component of cellular antioxidant defense (Hokamp and Nabity, 2016). GGT is unstable in untreated urine and their activity must be measured immediately after sampling (Paltrinieri et al. 2016)

Increase in urinary GGT:creatine ratio (uGGT/Cr) has been observed in pathological conditions leading to AKI. In dogs with pyometra, its increment was correlated with the severity of the histological lesions in the proximal renal tubules (De Loor et al. 2013). A slight to marked (2-3 folds) increase in uGGT/Cr has been reported in gentamicin and cisplatin experimentally induced nephrotoxicosis respectively, related to tubular injury (De Loor et al. 2013).

In dogs with natural renal disease was demonstrated that uGGT/Cr could be useful to detect established AKI, but not CKD (De Loor et al. 2013). Also, uGGT values were not useful to differentiate between non-azotemic and healthy dogs (Palacio et al. 1997).

One study demonstrated that high levels of uGGT/Cr could predict the presence of proteinuria in dogs with CKD secondary to *Leishmania* and could be helpful to differentiate between tubular and mixed proteinuria determined by urinary SDS-AGE (Ibba et al. 2016). It has been

suggested that the increase in uGGT/Cr levels could be useful to evaluate renal disease progression in CanL (Ibba et al. 2016), since tubular bands in urine electroforesis probably indicate more advanced renal lesions (Zatelli et al. 2003). In contrast, another study reported that changes in uGGT/Cr were not significant neither correlated to proteinuria improvement after 1 month of treatment (Pardo-Marín et al. 2017) probably due the presence of mainly unclassified mixed proteinuria.

7.2.2.2. N-acetyl-β-D-glucosaminidase (NAG)

N-acetyl-β-D-glucosaminidase (NAG) [150 kDa] and β-glucoronidase are lysosomal enzymes of the proximal tubular renal cells (Hokamp and Nabity, 2016). It seems that uNAG is not affected by age in dogs, but in humans it is lower in children than in adults, probably due changes in muscular mass and consequently in creatinine excretion (Smets et al. 2010). NAG is unstable in untreated urine and their activity must be measured immediately after sampling (Paltrinieri et al. 2016)

Dogs with lower urinary tract infection accompanied by pyelonephritis had markedly increased uNAG:creatine ratio (uNAG/Cr) values indicating probable tubular damage. Hematuria, pyuria, and bacteriuria/lower urinary tract infection without concomitant pyelonephritis does not alter uNAG or uNAG/Cr (De Loor et al. 2013).

In humans, NAG has been described as a helpful biomarker to confirm established AKI and as a predictor of its severity and outcome (De Loor et al. 2013). The magnitude of uNAG increment has been positive correlated to lesions in proximal tubules in dogs with pyometra. A slight to marked (2-3 folds) increase in uNAG/Cr has been reported in gentamicine and cisplatin experimentally induced nephrotoxicosis,

respectively, related to tubular injury (De Loor et al. 2013).

Increment in uNAG/c concentration is secondary to tubular damage or increased lysosomal turnover related to an increased competition for tubular reabsorption secondary to glomerular proteinuria. Consequently, the contribution of tubular damage to the urinary excretion of this protein could be difficult to assess (Nabity et al. 2012).

In dogs with CKD, uNAG values were significantly different compared to healthy dogs (Smets et al. 2010). In XLHN has been observed a constant increase in uNAG values during mid to late stage of the disease that could be related to a constant level of tubular damage and/or to glomerular proteinuria (Nabity et al. 2012). One study in dogs with CKD and proteinuria, shown a strong correlation with histological glomerular damage without correlation with tubulointerstitial lesions, suggesting that uNAG/Cr could also be useful to detect glomerular dysfunction in chronic proteinuric nephropathies (Hokamp et al. 2016).

Also, increased uNAG/Cr has been significantly associated with immune complex glomerulonephropathies, that in combination with uIgM/c had a moderate sensitivity of 75 and 78% respectively, to predict this group of renal diseases (Hokamp et al. 2016).

In one study, an evident significant increment of NAG and β-glucoronidase has been observed in urine of non azotemic dogs with *Leishmania*, compared with non azotemic healthy dogs (Palacio et al. 1997). Another study in CanL at different stages of renal disease reported an increase in uNAG levels at diagnosis, and a significant decrease in uNAG concentration correlated with improvement of proteinuria values after 1 month of treatment. In both studies, it was suggested that the increase in uNAG levels was secondary to the glomerular damage often seen in CanL, in addition to tubular damage (Pardo-Marín et al. 2017).

7.2.2.3. Retinol-binding Protein (RBP)

Retinol-binding protein (RBP) is 21-kDa LMW plasma protein synthesized mainly in the liver but also in the kidney, lungs, spleen, brain, stomach, heart, and skeletal muscle. It is the principal carrier of retinol (vitamin A). The RBP-retinol plasmatic complex bounds to transthyretin (TTR) that transports thyroxine and retinol. TTR-RBP complex cannot pass the glomerular barrier because its high weight. When retinol has reached its target tissues, the affinity of RBP for TTR decreases. Free RBP can be filtered by the glomeruli and reabsorbed in the proximal tubule after its cellular catabolism (De Loor et al, 2013).

Loss of RBP in urine could be observed as consequence of decrease reabsorption of RBP secondary to tubular damage and/or competition for reabsorption in the presence of large amount of protein in glomerular damage. Loss of the TTR-RBP complex due glomerular disease could contribute to urinary RBP losses (Hokamp and Nabity, 2016).

Urinary RBP (uRBP) has been described as potential marker for proximal tubular dysfunction in humans and dogs. uRBP may be useful to predict the severity and outcome of AKI, and to predict the development of microalbuminuria and diabetic nephropathy in normoalbuminuric humans (De Loor et al. 2013).

Increased concentrations of uRBP has been observed in dogs with CKD. Also, increment in urinary RBP:creatinine ratio (uRBP/Cr) levels has been reported in urolithiasis and XLHN by Western blot analysis, ELISA, or both in comparison with healthy dogs (Hokamp et al. 2016).

However, the utility of RBP to identify early kidney dysfunction is still

contradictory. Some studies in dogs described the detection of uRBP before the onset of azotemia in XLHN and the correlation with the progression of the CKD in this disease (Nabity et al. 2012). In contrast, other study shows no significant differences in uRBP values, between healthy and non azotemic dogs in the presence of decrease GFR measured by exogenous plasma creatinine clearance (Hokamp et al. 2016). Nevertheless, it has been reported that RBP could provide prognostic information and predict the clinical course of renal disease better than the magnitude of proteinuria, sCr, and in some cases renal biopsy analysis in humans with CKD (Nabity et al. 2012).

Recently, uRBP/Cr has been studied in renal disease due to CanL, finding a significant decrease in uRBP/Cr levels associated to improvement of proteinuria, and after changes in baseline sCr and SDMA after one month of treatment. The decrease in uRBP/Cr concentration was higher than other biomarkers of tubular damage such as uNAG/Cr while monitoring the treatment. However, the changes in URBP/Cr between healthy and ill dogs were not significant in this study (Pardo-Marín et al. 2017).

7.2.2.4. Clusterin (Clus)

Clusterin (Clus) is a glycoprotein of LMW formed by 2 subunits, NA1 and NA2, of 40 kDa each one (García-Martínez et al. 2012). Clus paticipates in cellular protection against stress, lipids transport, cell aggregation, promotion and as apoptotic agent and regulator in complement cascade. When kidney injury occurs, this protein is released into urine. In rats, has been suggested that urinary Clus (uClus) allows to differentiate tubular from glomerular proteinuria. In contrast, in various studies uClus has been described as a non-specific renal biomarker, because its increment in regional peri-infart associated with subtotal nephrectomy, ischemia-reperfusion injury, and posturethral obstruction in rats (García-Martínez et al. 2012). Also, uClus values increment

before sCr in rats under gentamicin treatment. Findings suggest that uClus determination could be useful to recognize early renal tubular and kidney injury in the clinical context (García-Martínez et al. 2012).

7.2.3. Biomarkers of renal inflammation

7.2.3.1. C-reactive protein (CRP)

Canine C-reactive protein (CRP) has a molecular weight of 100-115 kDa, is formed by 5 subunits of 20 kDa each one, and is mainly produced by hepatocytes at a relative low rate and storage in the endoplasmic reticulum (Cerón et al. 2005; Raila et al. 2011). In humans, some peripheral blood mononuclear cells are capable to produce CPR (Raila et al. 2011).

CRP is a positive acute phase protein, but is also considered a primitive antibody due to its interaction with cell membrane components of microorganism. CPR promotes binding of complement to improve bacterial phagocytosis, induces the production of cytokines, inhibits chemotaxis and modulates neutrophil function (Cerón et al. 2005).

CPR levels seem affected by age because the inflammatory response in experimental bacterial infection is higher in young (3 months) and adults (18 months) than in younger dogs (1 month). No significant relation with sex has been reported. During pregnancy serum levels of CPR are increased due inflammatory response against embryonic implantation (Cerón et al. 2005).

Under pathological situations, a quick and pronounced increase in CPR is observed in diverse conditions such as infectious diseases, trauma, surgery, systemic inflammatory response syndrome, or

immunomediated diseases (Martínez-Subiela et al. 2013).
Increase in serum CRP values has been observed in dogs with renal disease compared to healthy ones. Dogs with overt proteinuria and uremia have the highest levels of CPR in CKD (Raila et al. 2011). Serum CRP has been proposed as a useful renal marker to detect glomerular dysfunction in dogs with pyometra and babesiosis (Martínez-Subiela et al. 2013).

On the other hand, increase in serum CRP has been reported in dogs naturally and experimentally infected by *Leishmania infantum* showing a high sensitivity to detect infected dogs without clinical signs. Also, the decrease in CPR levels seems useful to monitor the efficacy of treatment in CanL (Martínez-Subiela et al. 2013). CRP increase has been considered a risk factor for mortality in CanL (Silvestrini et al. 2014)

For CRP to appear in urine, its plasma concentration must be increased and the glomerular barrier must be sufficiently damaged to allow its filtration (Raila et al. 2011; Hokamp and Nabity, 2016). Low concentrations of urine CPR (uCPR) has been found in non azotemic, non proteinuric leishmaniotic dogs, because if the glomerulus is not altered, there is no loss of CRP in urine (Martínez-Subiela et al. 2013). Instead, increase in urine CRP:creatinine ratio (uCRP/Cr) has been described in CanL at different stages of renal disease, with tendency to proportionally increase according to the severity of the proteinuria (Martínez-Subiela et al. 2013; Pardo-Marín et al. 2017). A significant increase in uCRP/Cr ratio has been reported in non proteinuric non azotemic dogs meaning that this ratio could be a more sensitive marker to detect renal damage than sCr (Martínez-Subiela et al. 2013). Significant decrease in serum CPR and uCRP/Cr has been associated with improvement of baseline UPC values in CanL with renal disease

and proteinuria (Martínez-Subiela et al. 2013), after 1 month of treatment (Pardo-Marín et al. 2017).

It is probable that CRP is also synthesized in situ in the damage kidney if there is severe proteinuria, azotemia and when the renal injury is more severe. Its increase could reflect the inflammation not only in the renal tissue but in other organs and tissues secondary to immune complex deposition in CanL. In consequence, monitoring urinary CRP concentrations in CanL could be useful to detect and evaluate the possible associated kidney damage (Martínez-Subiela et al. 2013).

7.2.3.2. Ferritin

Ferritin is a high molecular weight (900kDa) protein produced primarily by the liver that serves as iron storage. It is a moderate acute phase protein and a marker of oxidative stress (Martínez-Subiela et al. 2014; Hokamp and Nabity, 2016).

Ferritinuria has been reported in humans with urinary neoplasia and severe nephritis secondary to systemic lupus erythematosus, (García-Martínez et al. 2015).

Iron profile alterations in *Leishmania* infection have been related mainly to inflammation. However, one study shown that the increase of serum ferritin along with the decrease of serum iron values, is associated with the mechanism involved in the growth and virulence of *Leishmania* protozoa. Also, has been observed that serum ferritin had a positive correlation with serum CRP, and a negative correlation with serum iron and transferrin, indicating that the iron unavailability produced by inflammation in CanL, could contribute to the presentation of chronic anemia. Chronic bleeding presented in CanL was also related to the

changes in the iron profile (Silvestrini et al. 2014)
On the other hand, higher levels of serum ferritin have been observed in CanL in proteinuric compared to non proteinuric and borderline proteinuric dogs. In humans, high levels of serum ferritin have been associated to proteinuria secondary to glomerular disease (Martínez-Subiela et al. 2014).

Ferritinuria has been evaluated in dogs with *Leishmania* at different stages of renal disease. Higher urinary ferritin:creatinine ratio (uFer/Cr) was described in animals with proteinuria without azotemia suggesting than urine ferritin could be used as an early marker of renal dysfunction than sCr. Comparison of proteinuric and non proteinuric animals has shown a higher uFer/Cr in evident proteinuric than non proteinuric dogs. These results suggest that uFer/Cr concentration increases in the first stages of glomerular lesion when proteinuria is produced (García-Martínez et al. 2015; Pardo-Marín et al. 2017). Also, a significant decrease in UFer/Cr has been associated with improvement of proteinuria in renal disease secondary to CanL, after 1 month of treatment with antimonials and allopurinol (Pardo-Marín et al. 2017).

7.2.3.3. Adiponenctin

Adiponectin is a LMW (30 kDa) protein with 244 aminoacids that belongs to the adipokines, a specific group of secretory proteins. Adiponectin is present in the bloodstream in three oligomeric forms: a LMW trimer, a IMW hexamer, and a HMW 12- to 18-mers. Adiponectin accounts for 0.01% of the total plasma proteins in humans (Heidari et al. 2015). In dogs is considered a negative acute phase protein in experimental acute inflammation (Tvarijonaviciute et al. 2011)

Adiponectin has anti-atherogenic properties reducing cardiovascular risk, and could influence insulin resistance and cardiovascular disease in

type 2 diabetes and obesity in humans. In humans, plasma adiponectin could be markedly increased in renal disease associated to glomerular damage (Heidari et al. 2015) Increase in serum adiponectin was associated to increase in albuminuria and lower glomerular filtration rate levels in CKD.

Also, adiponenctin could be an early marker of kidney injury in systemic lupus erythematosus (Tvarijonaviciute et al. 2012; Heidari et al. 2015). Difference in serum adiponectin levels between non azotemic and azotemic dogs has been observed in *Leishmania* infection, in the presence of proteinuria (Tvarijonaviciute et al. 2012).

High levels of urinary adiponectin were correlated to increasing levels of urinary albumin excretion in diabetic nephropathy in type 1 diabetes in humans. In CanL, increase in urinary adiponectin was correlated to proteinuria suggesting its possible use as a marker of kidney injury (Tvarijonaviciute et al. 2012). It is probable that rise in urinary adiponectin is secondary to glomerular capillary leak because of glomerular damage, and a reflection of early vascular damage due to loss of adiponectin from the endothelial lining of glomerular vessels (Tvarijonaviciute et al. 2012).

In addition to the biomarkers indicated there are several urinary biomarkers studied in renal disease, but still not evaluated in CanL that appear in tables 3 and 4.

Table 3. Urinary biomarkers of tubular dysfunction not studied in canine leishmaniosis.

Biomarker	Production localization	Type	Mechanism causing urinary excretion	Presentation	References
KIM-1	Proximal tubular cells	Glycoprotein	Increased production	AKI	De Loor et al. 2013 Hokamp et al. 2016 Ostermann and Joannidis, 2016
NGAL	Neutrophils, kidney, bronchus, stomach, small intestine, pancreas, prostate, thymus	LMW glycoprotein	Decreased reabsorption and increased production	AKI CKD	De Loor et al. 2013 Hokamp et al. 2016
THP	Epithelial cells of thick ascending limb of loop of Henle and distal convoluted tubule	Glycoprotein	Decreased production	CKD	DeLoor et al. 2013 Hokamp et al. 2016
Vitamin D binding protein	Liver	Protein	Decreased reabsorption	CKD	De Loor et al. 2013 and Hokamp et al. 2016
α1-microglobulin	Liver	LMW protein	Decreased reabsorption	CKD	De Loor et al. 2013 Hokamp et al. 2016 Ostermann and Joannidis, 2016
β2-microglobulin	Surface of every nucleated cell	Light chain protein of MHC class I	Decreased reabsorption	CKD	De Loor et al. 2013 and Hokamp et al. 2016 Ostermann and Joannidis, 2016
APP	Renal proximal tubular brush border enzyme	Enzyme	Released from brush border	AKI	De Loor et al. 2013 Hokamp et al. 2016
ALP	Renal proximal tubular brush border enzyme	Enzyme	Released from brush border	AKI	De Loor et al. 2013 Hokamp et al. 2016

LDH	Proximal tubule	Enzyme	Released from brush border	AKI	De Loor et al. 2013 Hokamp and Nabity et al. 2016
IL-2, IL-7, IL-18	Local (kidney) or systemic	Cytokines	Inflammation	AKI	De Loor et al. 2013 Hokamp and Nabity et al. 2016
Monocyte chemoattractant protein-1	Local (kidney) or systemic	Cytokine	Inflammation	AKI	De Loor et al. 2013 Hokamp and Nabity et al. 2016
Granulocyte macrophage colony-stimulating factor	Local (kidney) or systemic	Cytokine	Inflammation	AKI	De Loor et al. 2013 Hokamp and Nabity et al. 2016
Keratinocyte-derived chemokine	Local (kidney) or systemic	Cytokine	Inflammation	AKI	De Loor et al. 2013 Hokamp and Nabity, 2016
Apolipoprotein A1	Local (Kidney) or systemic	Protein	Inflammation	CKD	De Loor et al. 2013 Hokamp and Nabity, 2016
Cubilin	Proximal tubule and other tissues	Endocytic glycoprotein receptor	Decrease albumin endocytosis and increased urinary albumin excretion	CKD	Birn et al. 2000 De Loor et al. 2013 Hokamp and Nabity, 2016
Megalin	Proximal tubule and other tissues	Endocytic glycoprotein receptor	Decrease albumin endocytosis and increased urinary albumin excretion	CKD	Birn et al. 2000 De Loor et al. 2013 Hokamp and Nabity, 2016

*Adapted from Hokamp JA and Nabity MB. Renal biomarkers in domestic species. Vet Clin Pathol. 2016 Mar;45(1):28-56

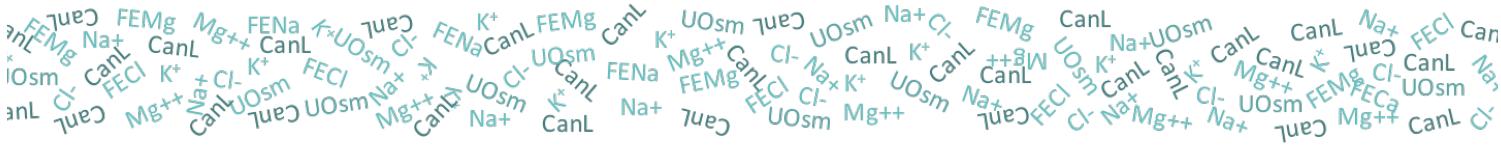
AAP, alanine aminopeptidase; AKI, acute kidney injury; ALP, alkaline phosphatase; CKD, chronic kidney disease; IL-2, IL-7, IL-18 = interleukin 2, 7 and 18, respectively; KIM-1, kidney injury molecule-1; LDH, lactate dehydrogenase; LMW, low molecular weight; MHC, major histocompatibility complex; NGAL, neutrophil gelatinase-associated lipocalin; THP, Tamm–Horsfall protein.

Table 4. Urinary biomarkers of glomerular dysfunction not studied in canine leishmaniosis.

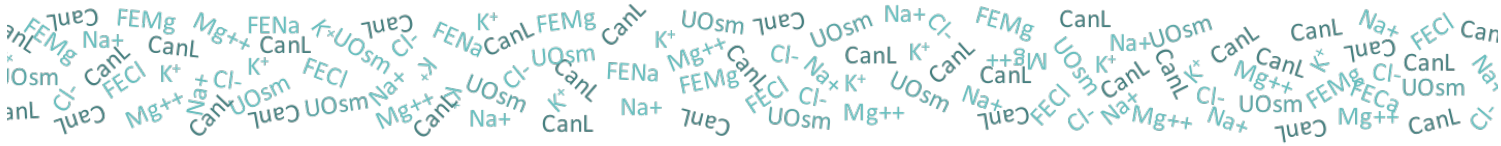
Renal biomarker	Production localization	Type	Clinical condition studied	References
Albumin	Hepatocytes	Negative acute phase protein	AKI CKD	Hokamp et al. 2016
TXB2	Glomerular mesangial cells and podocytes	Cyclooxygenase lipid metabolite; marker of altered intra-renal hemodynamics	AKI	Hokamp et al. 2016
Transferrin	Primarily liver; other tissues as well	Iron transport protein	CKD	Hokamp et al. 2016

*Adapted from Hokamp JA and Nabity MB. Renal biomarkers in domestic species. Vet Clin Pathol. 2016 Mar;45(1):28-56.

AKI, acute kidney injury; CKD, chronic kidney disease; TXB2, thromboxane B2.



8. Estudios



8.1. Estudio I

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Analytical validation and reference intervals for freezing point depression osmometer measurements of urine osmolality in dogs

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Abstract. Urine osmolality (UOsm) is considered the most accurate measure of urine concentration and is used to assess body fluid homeostasis and renal function. We performed analytical validation of freezing point depression measurement of canine UOsm, to establish reference intervals (RIs) and to determine the effect of age, sex, and reproductive status on UOsm in dogs. Clinically healthy dogs (n = 1,991) were retrospectively selected and stratified in groups by age (young [0–12 mo], adults [13–84 mo], and seniors [>84 mo]), sex (females and males), and reproductive status (intact and neutered). RIs were calculated for each age group. Intra- and inter-assay coefficients of variation were <1% in all cases. Good linearity (r^2 = 1, $p < 0.001$) and recovery (89–98%) were observed. The limit of detection and limit of quantification were zero. Urine specific gravity and UOsm had a highly significant positive correlation ($r = 0.96$, $p < 0.001$) but had inconsistent agreement. The 95% RI for canine UOsm was 369–2,416 mOsm/kg in young and adult dogs, and 366–2,178 mOsm/kg in seniors. Senior dogs had a significantly lower UOsm than young and adult dogs ($p < 0.000$). Neutered females had a significantly lower UOsm than intact female dogs ($p < 0.002$). These results indicate that the method evaluated is adequate for UOsm measurement and that RIs based on age and reproductive status should be used in dogs.

Key words: Dogs; osmolality; reference interval; urine concentration; validation studies.

Introduction

Urine concentration allows evaluation of renal response to variations in body fluid homeostasis and assessment of renal tubular function. Colorimetric reagent strips, refractometric specific gravity, and freezing point osmolality are used to measure urine concentration.[3] Test strips utilized to measure urine specific gravity (USG) are based on change of color of bromothymol blue used as the pH indicator.[3] In dogs, USG determined by test strips is considered unreliable because alkaline pH and glucosuria influence the measurement, giving lower results.[4] Refractometry provides an indirect estimate of USG by measurement of the urine refractive index, which is the ratio of the velocity of light in air to the velocity of light in solution. The number, mass, and chemical structure of dissolved particles in solution affect the index of light reflection and hence the measurement. The refractometer SG scale is based on experimental data from normal human, not canine, urine.[3,4,6]

Urine osmolality (UOsm) is considered the most accurate method to determine urine solute concentration in humans.[6] Osmolality is defined as the number of moles of osmotically active particles per kilogram of solution and is expressed in milliosmoles per kilogram (mOsm/kg). Osmolality is not influenced by the size, weight, or electric charge of particles, but by the number of osmolytes per unit of solvent.[1,2,18]

UOsm is usually measured by freezing point depression osmometry, based on the principle that each mole of dissolved solute will decrease the freezing point of a liquid by 1.86°C.[1] Most freezing point osmometers are automatic and quick, and measurements are simple to perform.[2] Clinically, decreased UOsm is considered an early marker of renal dysfunction.[12,22] Urine concentrating ability decreases in humans[13,19] and dogs[21] with age. This variation is attributed to a decrease in glomerular filtration rate and renal blood flow, and a failure of normal extrarenal or renal responsiveness to antidiuretic hormone (ADH).[13,19,21] Sex

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dimorphism is described in humans, with UOsm higher in men than in women.[15] Similarly, in a small population study, UOsm values were reported to be higher in male than in female dogs.[8]

We performed analytical validation of UOsm in dogs using an automated freezing point depression osmometer. In addition, we developed reference intervals (RIs) for age groups and evaluated effects of age, sex, and reproductive status on UOsm of healthy dogs.

Materials and methods

Clinically healthy dogs (n = 1,991) were selected retrospectively from 2008 to 2014 from the San Marco Private Veterinary Clinic (Padova, Italy) database, regardless of age, sex, reproductive status, or breed, to develop RIs for UOsm. Patients were considered clinically healthy after a normal physical examination and complete blood count, biochemistry panel, and urinalysis including USG, urine protein-to-creatinine ratio, and urine analytes within the in-clinic laboratory RIs. We did not perform further tests, such as abdominal ultrasound, to confirm the absence of early renal disease. In order to determine RIs for different age groups, dogs were stratified into 3 age groups: young (0–12 mo), adults (13–84 mo), and seniors (>84 mo). Ages ranged from 1 to 193 mo (mean 55). The dogs were of 130 different breeds. Most common breeds were mixed-breed (454), Labrador Retriever (189), and German Shepherd (111). The population consisted of 1,117 females (409 neutered and 708 intact) and 874 males (753 neutered and 121 intact; Table 1).

Urine samples were collected by ultrasound-guided cystocentesis, transferred to 5-mL sterile plastic vials, and analyzed within 1 h post-collection. Remaining samples were stored at −20°C. The UOsm measurement was performed by an automatic freezing point depression osmometer (Osmo Station OM-6050, ARKRAY, Menarini Diagnostics, Kyoto, Japan). The instrument was checked before use by 3 internal quality controls: human urine chemistry controls (Liquichek, Bio-Rad Laboratories, Hercules, CA) level 1 (446 mOsm/kg) and level 2 (804 mOsm/kg), and distilled water (0 mOsm/kg). Three-point calibration was performed with distilled water (0 mOsm/kg), and the low (300 mOsm/kg) and high (1,000 mOsm/kg) aqueous sodium chloride standard solutions levels provided by the manufacturer (ARKRAY, Menarini Diagnostics). Frozen urine was thawed at room temperature for 1 h and homogenized before measuring osmolality when used for validation studies.[4] All measurements were conducted by the same operator.

Three urine pools (low ≈ 400 mOsm/kg, medium ≈ 1,200 mOsm/kg, and high ≈ 2,000 mOsm/kg) were made to evaluate the precision, linearity under dilution, and limit of quantification (LOQ) of the method. Each pool was obtained by mixing 4 different urine samples. Five diverse urine samples with different UOsm were selected to perform the recovery experiment.

Table 1. Distribution of the dog population by sex, age, and reproductive status.

	Reproductive status	
Sex/Age group	Intact	Neutered
Female (n = 1,117)		
Young (n = 238)	217	21
Adults (n = 622)	402	220
Seniors (n = 257)	89	168
Male (n = 874)		
Young (n = 147)	146	1
Adults (n = 480)	426	54
Seniors (n = 247)	181	66

n = number of animals.

The intra-assay coefficient of variation (CV) was calculated based on 10 successive measurements on duplicates of the 3 urine pools. Inter-assay CV was calculated based on 10 consecutive runs in duplicate, carried out on 5 different days for each urine pool.[11]

Accuracy was evaluated based on a recovery test and linearity under dilution study.[14] Recovery was defined as the percentage increase of concentration that was measured in relation to the amount of the standard solution added.[11] Five urine samples with low values: 351 mOsm/kg (sample 1), 642 mOsm/kg (sample 2), 402 mOsm/kg (sample 3), 448 mOsm/kg (sample 4), and 550 mOsm/kg (sample 5), were selected for the recovery experiment. Two aliquots were prepared from each of these samples: the high UOsm standard solution (1,000 mOsm/kg) was added (1:1 volume) to the first aliquot. Distilled water (0 mOsm/kg) was added to the second aliquot (1:1 volume). All samples were analyzed in duplicate. Detected and expected UOsm levels were compared, and the recovery percentages were calculated as described previously.[23] Linearity under dilution was performed using a canine urine sample with known concentration (1,922 mOsm/kg) diluted at 0%, 10%, 20%, 40%, 50%, 60%, 90%, 95%, and 100%, using distilled water. Dilutions were analyzed consecutively in duplicate.[10] Linearity of the dilution series was assessed by linear regression.[23]

Limit of detection (LOD) was calculated as the median value plus 2 standard deviations (SD). LOD was based on 10 consecutive measurements in duplicate of the blank (distilled water).[11] LOQ was calculated as the lowest amount of analyte that could be measured with an intra-assay CV < 15%.[20] Twenty-six serial dilutions of 1 urine pool with high UOsm were prepared using distilled water and analyzed 4 times. The CV of each dilution was calculated and plotted as a function of UOsm concentration.

In order to evaluate the agreement between USG and UOsm methods,[11] USG was measured in all animals by a desktop clinical refractometer (T3-NE, ATAGO USA, Bellevue, WA) that was calibrated with distilled water (SG =

1.000). Intra-assay CV, inter-assay CV, and recovery were calculated using routine statistical procedures[11,23] with statistical software (Analyse-it v.3.5, Analyse-it Software, Leeds, UK; MedCalc v.14.8.1, MedCalc Software, Ostend, Belgium). Regression analysis was used to compare measured and expected UOsm values obtained in the linearity under dilution study. Method comparison between UOsm and USG was studied by Spearman regression, Passing–Bablok regression, and Bland–Altman plot using commercial software (Analyse-it v.3.5; MedCalc v.14.8.1). Mean and 95% confidence interval (CI) were used to define RIs. All variables met normal distribution criteria and were evaluated with paired 2-tailed Student *t*-test. Comparison between age, sex, and reproductive status was performed using ANOVA with a Tukey contrast test for multiple comparisons. Statistical significance was set at $p < 0.05$ for all analyses.

Results

Intra-assay and inter-assay CVs were 0.23–0.89% and 0.16–0.8%, respectively (Table 2). Serial dilutions of a urine pool with high UOsm resulted in linear regression equations with an adjusted correlation coefficient (r^2) of 1, and a Pearson coefficient $p < 0.001$ that reveals an excellent fit to the linear model. Intercept of 2.87 (–5.96 to 11.69) with 95% CI did not differ significantly from zero. Slope was 19.41 (19.27–19.55; Fig. 1). Recovery was 89–98% between observed and expected UOsm (mean: 94.8%; Table 3). The LOD was zero (0 ± 0 mOsm/kg), and the assay was precise with any value tested, therefore the LOQ was also zero. UOsm and USG showed a highly significant positive correlation ($r = 0.96$, $p < 0.001$). A proportional error was observed between the methods (Fig. 2).

The 95% UOsm RI for young and adult dogs was 369 (CI: 316–395 mOsm/kg) to 2,416 mOsm/kg (CI: 2,364–2,482 mOsm/kg). In senior dogs, UOsm RI was 366 (CI: 342–405 mOsm/kg) to 2,178 mOsm/kg (CI: 2,032–2,285 mOsm/kg; Table 4). Senior dogs had a significantly lower UOsm ($p < 0.000$) than young and adult dogs (Table 5).

No statistically significant differences were found between males and females in any age group in the population. UOsm was significantly lower in neutered (1,278 ± 530 mOsm/kg) than in intact female dogs (1,386 ± 569 mOsm/kg; $p < 0.002$). No statistically significant differences were found in UOsm between neutered and intact male dogs (Table 6).

Discussion

Precision and linearity under dilution in our study are similar to those reported in humans[5] and dogs.[21] The recovery value was better in the lower range in our study than in the human one.[5] However, in the human study, controls and standard solutions were used for validation instead of urine pools.

Table 2. Intra-assay and inter-assay coefficients of variation (CVs) of urine osmolality (UOsm) determined by freezing point depression osmometry in canine urine pools with 3 concentration levels (low, medium, high).

Comparison/Pool	Mean UOsm (mOsm/kg)	SD	Mean CV (%)
Intra-assay			
Low	401	0.92	0.23
Medium	1,176	4.88	0.41
High	1,891	16.97	0.89
Inter-assay			
Low	382	0.64	0.16
Medium	1,177	4.15	0.34
High	1,873	15.17	0.80

SD = standard deviation.

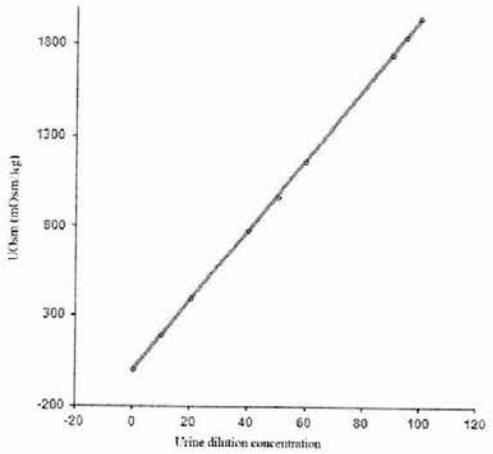

Figure 1. Linearity under dilution from 9 samples with different concentrations, after serial dilution with distilled water of 1 canine urine sample (1,992 mOsm/kg). Urine osmolality (UOsm) was determined by freezing point depression osmometry. Each point is the mean value of duplicate determinations.

The comparison between USG by refractometry and UOsm by freezing point depression showed a highly significant positive correlation in our study, similar to previous reports in dogs.[1,4] Nonetheless, some analytes have been found to cause interference and may influence this relationship, such as ketones in the dog,[1] and ketones, protein, and glucose in humans.[16,17] We observed a proportional error between the methods with extreme values having the highest differences, making the methods not comparable. Results in human patients with renal impairment suggest that the relationship between USG and UOsm is not as consistent as expected, but the reason is unclear.[17]

We found the osmometer validated for our study to be trouble-free and simple to use. Furthermore, its automatic

Table 3. Recovery percentages from 5 different canine specimens with low urine osmolality (UOsm), measured by freezing point depression osmometry, after preparing 2 aliquots from each specimen by adding different solutions.

Specimen	Expected value (mOsm/kg)		Observed value (mOsm/kg; duplicate mean)		Recovery (%)
	Sample A	Sample B	Sample A	Sample B	
1	451	319	456	360	96
2	742	577	708	619	89
3	502	361	503	405	98
4	548	400	548	451	96
5	630	477	620	524	95

The first aliquot (sample A) was prepared by adding a high UOsm standard. The second aliquot (sample B) was made by adding distilled water.

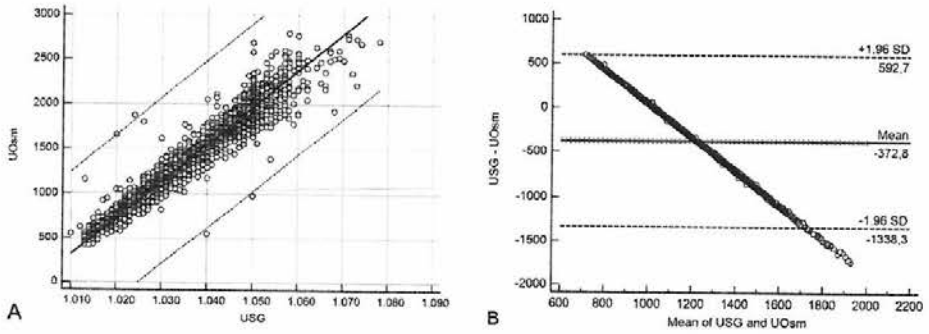

Figure 2. A. Passing–Bablok regression plot for urine osmolality (UOsm; mOsm/kg) and urine specific gravity (USG) in healthy dogs (n = 1,991). The solid line represents the regression line (y = 41.2x–41,316). The 2 dashed lines indicate confidence bands of the regression line (intercept 95% CI: −41,789 to −40,856; slope 95% CI: 40.8–41.7). The dotted line is the identity line; Pearson correlation coefficient is $r = 0.96$, $p < 0.001$. The Passing Bablok regression indicates an inconsistent relationship and a high constant and proportional difference between methods. **B.** Bland–Altman scatter diagram of the differences plotted against the average of UOsm and USG measurements. The solid line represents the mean difference, and the dash-dotted lines on either side are its 95% CI. The dashed lines are the limits of agreement, defined as the mean difference ±1.96 times the standard deviation (SD) of the differences.

Table 4. Urine osmolality reference intervals in young and adult, and senior dogs.

Age group	Mean reference interval (mOsm/kg)*	95% confidence interval (mOsm/kg)
Young and adult	1,408 (369–2,416)	316–395 to 2,364–2,482
Senior	1,204 (366–2,178)	342–405 to 2,032–2,285

* Numbers in parentheses are 95% confidence intervals.

Table 5. Urine osmolality (UOsm) values in different age groups of healthy dogs.

Variable/Group	n	Mean UOsm (mOsm/kg)	SD	p value
Age				
Young	385	1,437	592	
Adult	1,102	1,398	538	
Senior	504	1,204	490	0.000*

n = number of animals; SD = standard deviation.
*Statistically significant versus young and adult dogs.

processing and technical characteristics make it suitable for clinical settings, as well as for laboratories with large volumes of samples.[5,9]

Our results agree in part with those of 2 other studies of UOsm RIs in dogs,[7,21] although the number of animals included in the previous studies was considerably smaller than in our study. The upper value of RIs in our study is similar to that elsewhere (2,546 mOsm/kg),[7] but slightly lower than that observed by others (2,830 mOsm/kg).[21] More controversy exists in the lower limit of the RIs. Our values are between those described by 2 other studies (976 mOsm/kg[7]; 161 mOsm/kg[21]). The difference between studies can be attributed to the different populations evaluated and the number of animals included.

In our study, UOsm was significantly lower in senior than in young and adult dogs, confirming the decrease of urine concentration in older dogs as described previously.[21] In humans[13,19] and dogs,[21] decreased capability to concentrate or dilute urine with age is related to a reduction in glomerular filtration rate and renal blood flow, failure of normal extrare-

Table 6. Comparison between urine osmolality (UOsm) and reproductive status in young, adult, and senior female and male dogs.

Variable	Sex	Age group	Reproductive status	Mean UOsm (mOsm/kg)	± SD	*p* value
Sex	Female	Young	Neutered	1,359	502	0.49
			Intact	1,452	635	
		Adult	Neutered	1,370	546	0.87
			Intact	1,378	545	
		Senior	Neutered	1,149	487	0.07
			Intact	1,264	479	
	Male	Young	Neutered	1,797	*	*
			Intact	1,422	538	
		Adult	Neutered	1,330	494	0.15
			Intact	1,440	530	
		Senior	Neutered	1,265	521	0.59
			Intact	1,202	485	

SD = standard deviation.
* SD and statistical comparison between young male neutered and intact dogs could not be performed because there was only one neutered animal in the age group evaluated.

nal or renal responsiveness to ADH, impaired thirst mechanism, and increase in sodium losses.

Differences between morning and evening UOsm values have been reported in dogs.[21] However, urine samples were randomly collected throughout the day in our study. Nonetheless, our results are representative of the situations that occur in routine clinical practice where urine can be collected at different times of the day.

In humans, men have higher UOsm values than women.[15] It seems that this difference does not depend on the direct effects of sex hormones or on the level of sodium intake.[15] Higher UOsm in men could be attributed to the fact that men concentrate urine more than women on their usual diet and spontaneous fluid intake.[15] Similar findings has been reported in male dogs.[8] In our study, no statistically significant differences were observed by sex. However, significantly higher UOsm values in intact than in neutered females were observed. To our knowledge, there is no evidence that can explain these results.

Acknowledgments

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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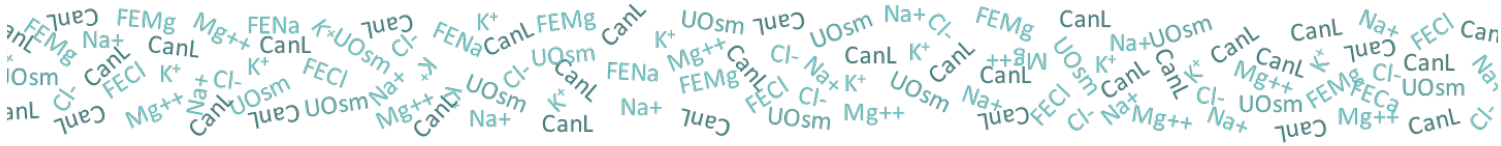

8.2. Estudio II

Urine osmolality and fractional excretion of electrolytes in dogs with canine leishmaniosis.

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Abstract

Background: Renal disease is one of the main complications of canine leishmaniosis. The objectives of this study were to evaluate urine osmolality and fractional excretion (FE) of Na, K, Cl and Mg in dogs with leishmaniosis at different stages of renal disease and to determine the changes of these biomarkers after one month of treatment. These analytes were measured in thirty-five dogs distributed in 4 groups. Group 1: non proteinuric, non azotemic; Group 2: borderline proteinuric, non azotemic; Group 3: proteinuric, non azotemic; Group 4: proteinuric and azotemic. In addition, changes in these analytes were evaluated after therapy in 15 dogs.

Results: FENa, FECl and FEMg increased significantly as renal disease progressed in canine leishmaniosis. FEMg significantly decreased in dogs after successful treatment.

Conclusions: FENa, FECl and FEMg values could be useful to detect early tubular dysfunction before the presentation of azotemia in renal disease secondary to canine leishmaniosis. Furthermore, FEMg could be useful for leishmaniosis treatment monitoring in dogs.

Keywords: dog, fractional excretion, FECl, FEK, FEMg, FENa, leishmaniosis, proteinuria, renal disease, urine osmolality**.**

Abbreviations

AKI, acute kidney injury; ATN, acute tubular necrosis; CKD, chronic kidney disease, CanL, canine leishmaniosis; FE, fractional excretion; FECl, fractional excretion of Cl; FEK, fractional excretion of K; FEMg, fractional excretion of Mg; FENa, fractional excretion of Na; FEs, fractional excretions; GFR, glomerular filtration rate; sCr, serum creatinine; Se, serum electrolyte concentration; uCr, urinary creatinine; Ue, urinary electrolyte concentration; UPC, urine protein:creatine ratio;

UOsm, urine osmolality.

Introduction

Canine leishmaniosis (CanL) caused by *Leishmania infantum*, is transmitted by the bite of phlebotomine sand flies. CanL is endemic in the European Mediterranean Region, Asia, North Africa and South America affecting millions of dogs, and it is an emergent disease in North America (Duprey et al. 2006; Dujardin et al. 2008). Chronic kidney disease (CKD) is commonly seen in CanL associated with glomerulonephritis (Solano-Gallego et al. 2011). Interstitial nephritis has been also described in CanL (Costa et al. 2003). Moreover, in human leishmaniosis, tubular dysfunction has been reported (Lima Verde et al. 2007; Oliveira et al. 2011), Asymptomatic, slow and progressive development of renal disease often seen in CanL, points out the necessity to identify new early biomarkers able to detect early kidney injury and to evaluate the progression of the disease and the response to treatment (Solano-Gallego et al. 2011).

Tubular integrity and function can be evaluated by urine osmolality (UOsm) and by fractional excretion (FE) of various electrolytes. UOsm is considered one of the best ways to evaluate the ability of the tubules to concentrate urine (George, 2001) especially under pathologic conditions (Sethi et al. 2010; Ayoub et al. 2013). Recently, UOsm measured by freezing point osmometry has been validated and reference intervals for young-adult, and senior dogs have been described (Guerrero et al. 2017). To the author's knowledge few studies have evaluated UOsm in dogs with renal disease (Ayoub et al. 2013).

In addition, the ability of the tubules to handle electrolytes can be evaluated by the calculation of FE of different electrolytes such as Na,

Cl, K or Mg, that reflects the amount of filtered particles that escapes reabsorption and are excreted in urine (Lefebvre et al. 2008). FEs are affected in cases of tubular renal impairment (Chan et al. 2002; Schier, 2011, Alsaad et al. 2016). Spot sample measurement of FEs is a simple, non-invasive, and cost-effective diagnostic tool to assess renal tubular function in dogs (Brown et al. 2015). In humans, FEs have been studied in different diseases and some specific changes have been described. Increment in FENa was useful to determine tubular damage in oliguric acute tubular necrosis [ATN] (Carvounis et al. 2002) and FEK increases at the end stage renal disease to understand K homeostasis in CKD (Ueda et al. 2016). In addition, FEMg has been used to assess tubular damage in ATN and at early stages of CDK in humans (Gheissari et al. 2011), and it has been proposed as an indicator of the degree of tubular cell injury in dogs (Buranakarl et al. 2007).

In dogs, increases in FE of Na, K, Cl and Mg have been reported as consequence of tubular reabsorption defects in spontaneous Fanconi's syndrome in dogs (Lefebvre et al. 2008). Also, FENa increases before serum creatinine (sCr) changes, were described in acute tubular damage induced by gentamicin associated to ultrastructural changes in proximal renal tubules (Lefrebvre et al. 2008). FEMg increases at the beginning of AKI due to tubular dysfunction and the tendency of urinary fractional clearance of Mg to decrease before its successful treatment, have been observed as well (Brown et al. 2015). Likely, reduction in FENa and FEK concentrations were associated with improvement of tubular function and correlated with survival in AKI (Brown et al. 2015). On the other hand, in CDK and partially nephrectomized dogs, higher values of FENa and FEK have been reported (Lefrebvre et al. 2008). To the author's knowledge no data has been published about UOsm in dogs naturally infected with *Leishmania* and only one study evaluated some FE of electrolytes in CanL (Sousa et al. 2016).

The objectives of this study were to evaluate UOsm and FE of Na, K, Cl and Mg in dogs with leishmaniosis at different stages of renal disease, and their possible changes after treatment.

Material and Methods

Animals

Thirty-five dogs (*n*=35) naturally infected with *L. infantum* from different clinics of southern Spain were included in this prospective study from 2014 to 2015. CanL diagnosis was established based on compatible clinical and/or laboratory findings and the presence of high antibody titers for *L. infantum* measured with a SNAP test (Leiscan® Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) and the identification of the parasite by positive real-time PCR from bone marrow or lymph node samples and/or by direct visualization on cytology examination. All animals were tested negative for co-infections with Canine Heartworm, *Anaplasma phagocytophylum*, *Borrelia burgdorferi*, and *Ehrlichia canis* using a SNAP test (SNAP® 4DX kit, IDEXX Laboratories Inc., Westbrook, ME).

Dogs with active urine sediment, at risk of developing glomerulopathy associated with concomitant diseases and in treatment with angiotensin-converting enzyme inhibitors or any other drug in the previous 6 months before the beginning of the study, were excluded.

Dogs were distributed in four groups according to a previous study (Martínez-Subiela et al. 2013). Group 1: non proteinuric, non azotemic (n=10); Group 2: borderline proteinuric, non azotemic (n=11); Group 3: proteinuric, non azotemic (n=10); Group 4: proteinuric and azotemic (n=4). Dogs were considered non proteinuric if urine protein:creatinine ratio (UPC) was <0.2, borderline if UPC ratio was 0.2–0.5, and

proteinuric if UPC was >0.5. Azotemia was defined as sCr>1.4 mg/dL.

The samples were collect from all dogs on the day of the diagnosis before any treatment was initiated. In addition, follow-up was performed in 15 animals evaluated at initial diagnosis and after 4 weeks of treatment with N-methylglucamineantimoniate (50 mg/kg SC, two times daily) and allopurinol (10 mg/kg PO BID).

Treated dogs were divided in Group A (n=6): proteinuric dogs before treatment and with an increase or without decrease of UPC baseline values after treatment, and Group B (n=9): proteinuric dogs before treatment with decrease UPC baseline values after treatment.
The study was approved by the Research Ethics Committee of Murcia University.

Sample collection
Blood samples were collected from the cephalic vein, conserved in serum clot activator tubes and allowed to clot at room temperature. Serum was obtained by centrifugation (3000 g, 10 min) and stored at –80 °C until analysis.
Urine samples were collected by ultrasound-guided cystocentesis (5 mL, 22-G needles) and centrifuged (300 g, 2 min). Urine sediment analysis was performed to exclude samples with active sediment. The supernatant was stored at −80ºC until analysis.

UOsm and FEs measurement

UOsm (mOsm/kg) measurement was performed using an automatic freezing point depression osmometer (Osmo Station OM-6050, ARKRAY, Menarini Diagnostics, Kyoto, Japan).

Spot urine and plasma samples were used for FE of electrolytes determination. FE of Na, K, Cl and Mg were calculated following the mathematical equation, where "Ue" corresponds to a determined urine electrolyte concentration, "Se" to the serum concentration of the same electrolyte and "sCr and uCr", to serum and urine creatinine concentration respectively:

FE= (Ue x sCr)/(Se x uCr)

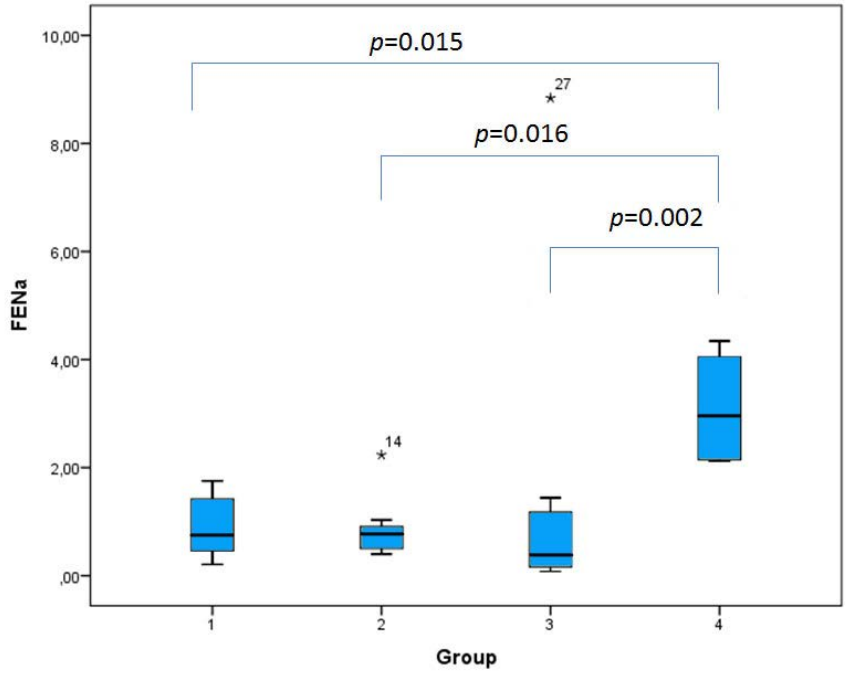
Statistical analysis

Statistical analysis was performed with a statistical software (IBM SPSS Statistics 22.0. New York, NY) using a Kolmogorov-Smirnov test to determine if variables were non-parametric. A Kruskal Wallis analysis was used to study variables in different stages of renal disease. Wilcoxon test was applied to compare variables after and before treatment. Statistical significance was set a p=<0.05.

Results

Results of FE of different electrolytes and UOsm in the different groups of dogs affected by CanL appear in Fig. 1. FENa was significantly higher in Group 4 than in group 1 (p=0.015), group 2 (p=0.016) and group 3 (p=0.002). FECl was significantly higher in group 4 than in group 1 (p=0,012), group 2 (p=0.014) and group 3 (p=0.002). FEMg was significantly higher in Group 4 than in group 1 (p=0.005), group 2 (p=0.02) and group 3 (p=0.015).

A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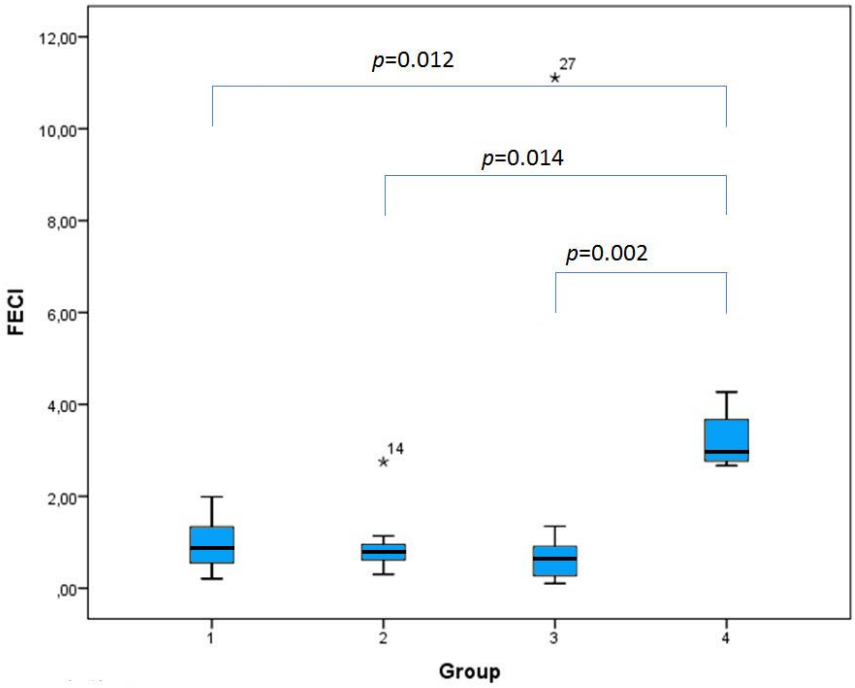
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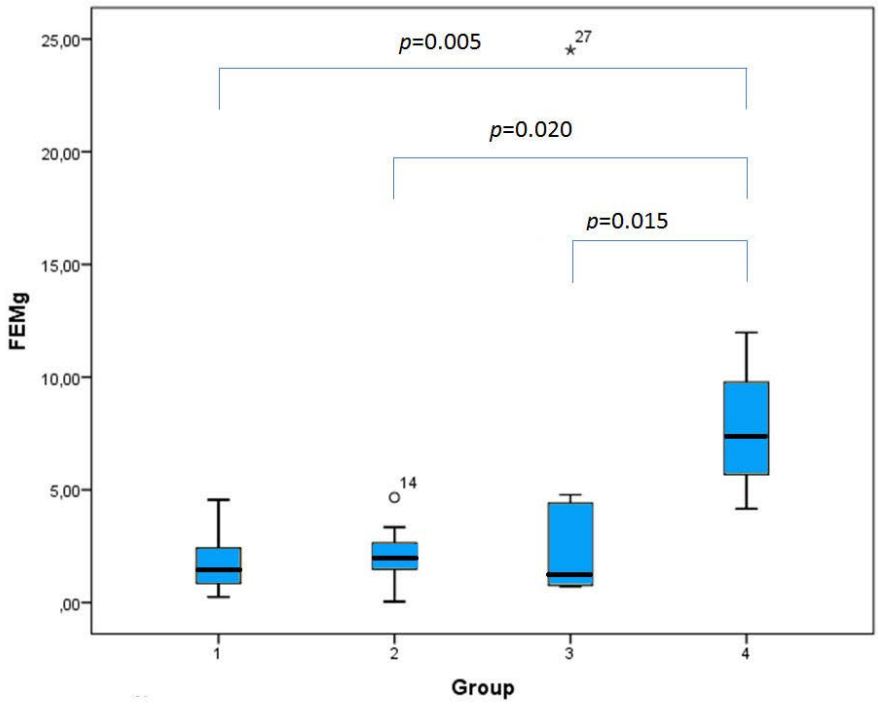

Figure 1. A. FENa **B.** FECl and **C.** FEMg in 4 groups of dogs (Group 1=non proteinuric, non azotemic; group 2=borderline proteinuric, non azotemic; group 3=proteinuric, non azotemic; group 4=proteinuric and azotemic) at different stages of renal disease secondary to leishmaniosis. The boxes depict median (horizontal line) and interquantile range (top and bottom of the box). The whiskers show the 25% and 97.5% range, and outliers are represented as separated points.

There were not significant differences in FEK and UOsm values between groups, although FEK and UOsm showed tendency to increase and decrease in group 4, respectively.

FEMg was significantly lower (p=0.046) one month after treatment in all animals in the follow-up group. There was no significant difference between Group A and Group B in any of the FEs evaluated neither in the UOsm values, although UOsm showed tendency to improve. Results of FE of different electrolytes and UOsm before and after treatment appear in Table 1.

Table 1. Comparison of FE Na, K, Cl and UOsm in proteinuric dogs with leishmaniosis and renal disease with maintenance/worsening (Group A; n=6) or improvement (Group B; n=9) on proteinuria values, before (initial) and after one month of treatment (follow-up).

Analyte/Group		Group A (n=6)	Group B (n=9)	All animals (n=15)
FENa %	Initial	1.02 (0.52-4.27)	0.39 (0.23-7.51)	0.86 (0.29-7.27)
	Follow-up	1.04 (0.54-2.53)	1.02 (0.56-1,73)	1.02 (0.52-2.34
FEK %	Initial	18.92 (9.79-31.61)	17.41 (9.79-31.67)	12.59 (10.14-82.34)
	Follow-up	12.59 (10.40-90.389)	13.97 (13.03-42,70)	14.36 (12.25-41.44)
FECl %	Initial	0.90 (0.78-4.12)	0.54 (0.30-9.46)	0.89 (0.42-8.72)
	Follow-up	1.15 (0.62-2.34)	1.29 (0.81-3.06)	1.29 (0.64-2.96)
FEMg %	Initial	3.86 (1.47-11.38)	1.92 (0.80-21.12)	2.94 (0.89-20.12)
	Follow-up	2.90 (1.53-7.70)	2.44 (1.95-4.78)	2.69* (1.88-7.14)
UOsm (mOsm/Kg)	Initial	917.0 (617.50-1593.4)	705.0 (609.0-1694.4)	814.0 (580.5-1684.8)
	Follow-up	1101.5 (849.8-1355.0)	816.0 (641.0-1362.6)	937.0 (709.0-1369.6)

Values were represented as median (percentile 25-97.5%).

* $p<0.05$ vs. Initial within the group.

Discussion

This study evaluated the UOsm and FE of Na, K, Cl, Mg in 35 dogs naturally infected with *L. infantum* at different stages of renal disease, and reported the changes of these analytes in 15 dogs, before and after

one month of treatment. To the author's knowledge this is the first study in which UOsm and FE of Cl and Mg are evaluated in CanL.

Increment of FENa, FECl and FEMg has been related to renal tubular dysfunction in human leishmaniosis without azotemia with hypomagnesemia and hyponatremia (Silva Junior et al. 2014). In humans, hypomagnesemia has been related to increase in the risk of mortality in AKI and it is an indicator of progression of CKD (Noiri et al. 2015). FEMg rises in early stages of CKD [stage 1 and 2] (Gheissari et al. 2011) and it increases progressively to maintain normal serum Mg concentration until advance CKD (Felsenfeld et al. 2015). Also, high FENa values without azotemia has been reported probably related to renal damage in CanL (Sousa et al. 2016). In dogs with CKD, high values of FENa and FECl have been observed (Lefebvre et al. 2008), and their increase along with other FE of electrolytes during azotemia progression, could indicate the severity of renal dysfunction (Buranakarl et al. 2007). In agreement with previous data, all FEs evaluated in this study with exception of the K, presented significantly higher values only in those dogs with evident alteration of renal function and overt azotemia. However, serum electrolytes had no significant differences in the population studied (data not shown).

The absence of significant differences in FEK results would agree with previous reports in which no changes in FEK were observed in CKD, probably because the decrease in tubular Na reabsorption adaptively enhanced flow in residual nephrons, maintaining normal values of FEK (Ueda et al. 2016).

UOsm values begin to decrease when there is a 33% of tubular damage, being considered an early marker of renal tubular dysfunction

in humans (Bockenhauer and Aitkenhead, 2011; Ostermann et al. 2016) and dogs (De Loor et al. 2013; Waldrop, 2008). One study reported urine hyposmolality without azotemia or changes in GFR in humans with tubular renal dysfunction secondary to leishmaniosis, probably because of lower expression of aquaporine 2 and a compensatory increased expression of cotransporter N-K-2Cl, both involved in urine concentration/dilution renal mechanisms (Oliveira et al. 2011). In our study, UOsm shown tendency to decrease with the progression of the renal disease in CanL and to increase after treatment, but the results were not statistically significant. Further studies in a larger population would be recommended to evaluate the role of UOsm in renal disease and CanL.

The results obtained after monitoring of treatment would indicate that changes in FE of electrolytes and UOsm are not associated with the evolution of proteinuria, probably because the proteinuria is more related with glomerular damage whereas these analytes are considered as tubular markers. Only FEMg showed a significant decrease after treatment. FeMg has been considered a better marker of renal tubulointerstitial dysfunction in humans, because Mg homeostasis depends mainly on its renal tubular reabsorption unlike Na, K and Cl urinary concentration (Felsenfeld et al. 2015; Noiri et al; 2015).

The lack of significance in most of the results after treatment could be due to the low number of dogs included. Further large scale studies should be done to evaluate these analytes in dogs with CanL.

Although this study should be considered a pilot report due to the small population evaluated, our findings demonstrate that increases in FE of Na, Cl and Mg are associated with the development of azotemia in CanL

and it could indicate early renal damage. FEMg evaluation could be useful to monitor therapy.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. However, if any additional information is required this can be obtained from the corresponding author on reasonable request.

Authors' contributions

SG, JP, JJC, SMS, AT designed the research and wrote the manuscript; SG, JP, JJC, SS, LP, SMS, AT, contributed to sample collection, analysis and interpretation of the data and revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of Murcia University.

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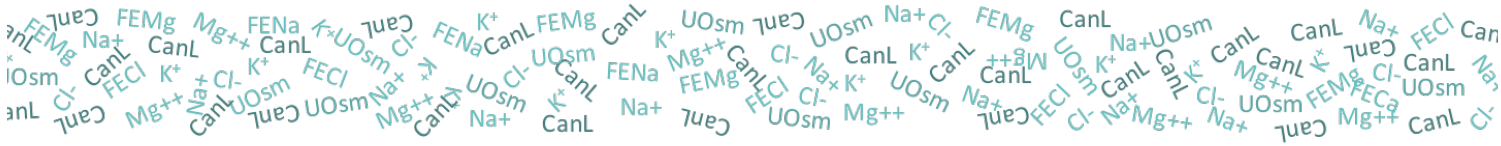


9. Discusión General

La capacidad de concentración del riñón es un proceso complejo influenciado por diversas variables como el mantenimiento del flujo sanguíneo renal, la carga de solutos, la presencia de la ADH y de la funcionalidad de sus receptores, la presencia de los transportadores de la urea y de las acuaporinas, y la correcta función del sistema de contracorriente renal. La concentración urinaria juega un papel fundamental en el balance hídrico y en la excreción de sodio (Sands and Layton, 2009), y puede verse afectada en los estadios iniciales de la enfermedad renal asociada principalmente con la disfunción tubular.

En perros con leishmaniosis se ha hecho hincapié en el estudio del daño glomerular, pero son escasos los estudios acerca de la presentación clínica de la disfunción tubular renal. Siendo la enfermedad renal una de las causas más importantes de mortalidad en CanL, es necesario encontrar biomarcadores que permitan detectar el daño renal incipiente e identificar el compromiso tubulointesticial del mismo, para implementar las medidas de prevención y acciones terapéuticas adecuadas.

La UOsm es considerada el parámetro de referencia para evaluar la concentración urinaria y valorar la homeostasis hidroelectrolítica en personas y en perros (Chadha et al. 2001; Dossin et al. 2003). Los cambios en la UOsm son útiles para detectar disfunción renal a nivel tubular (Manz ad Wentz, 2003; Waldrop et al. 2008) y para diferenciar la azotemia pre-renal de aquella de origen renal, en situaciones de hipovolemia o de hipoperfusión (Ostermann and Jannidis, 2016).

En esta investigación se realizaron dos estudios. En el **primer estudio** se validó por primera vez, la medición de la UOsm en perros, mediante el uso de la osmometría por punto de congelación. Así mismo, se establecieron intervalos de referencia en perros jóvenes-adultos, y en

perros ancianos. Además, se evaluó el efecto de la edad, el sexo y el estado reproductivo sobre la UOsm. En el **segundo estudio** se evaluó la UOsm y las FEs de Na, K, Cl y Mg en perros con leishmaniosis en diferentes estadios de la enfermedad renal, así como los cambios de dichos analitos después del tratamiento.

En el primer estudio de esta investigación se demostró que la osmometría de punto de congelación es un método preciso y eficaz, y con buenas características técnicas. Nuestros resultados fueron concordantes con los datos obtenidos en estudios anteriores realizados en humanos (Elorza et al. 1993) y en perros (van Vonderen et al. 1997). La validación analítica de este método en perros era inexistente, pero necesaria para permitir la implementación de la medición de la UOsm en el ámbito clínico e investigativo.

Aunque en la práctica clínica comúnmente se utiliza la USG medida por refractometría para estimar la concentración urinaria, la correlación de este método con la UOsm sólo es positivo en las muestras de animales sanos, cómo se demostró en el primer estudio de esta investigación, con resultados semejantes a los de estudios previos en perros (Dossin et al. 2003; Ayoub et al. 2013) y en humanos (Souza et al. 2015). Es necesario señalar que, en el caso de muestras patológicas el método de elección es la UOsm, ya que no se ve afectada por moléculas como las proteínas (Sethi et al. 2010; Souza et al. 2015) que pueden encontrarse en la orina en la CanL.

En la enfermedad renal secundaria a CanL, se ha observado que la USG está significativamente correlacionada con la GFR, pero que su capacidad para predecir las alteraciones de la misma es poca (Cortadellas et al. 2008). Teniendo en cuenta estos dos factores, podría

sugerirse que el empleo de la UOsm para determinar la concentración urinaria en la CanL es más apropiado.

La determinación de los límites mínimo y máximo de la UOsm en lugar de "valores normales", es importante para la interpretación de la concentración urinaria según el contexto clínico y valorar si la respuesta renal observada es la esperada para el mantenimiento de la homeostasis hídrica (Bockenhauerand y Aitkenhead, 2011). En el primer estudio de esta investigación obtuvimos los intervalos de referencia de la UOsm, con diferencias en el límite inferior con los estudios realizados anteriormente en perros (Hardy and Osborne, 1979; van Vonderen et al. 1997). Esta discrepancia podría ser consecuencia del número de animales incluidos en cada estudio, la diversidad de las poblaciones, y el efecto del ciclo circadiano, la dieta y el ejercicio sobre la concentración urinaria (van Vonderen et al. 1997; Manz and Wentz, 2003). Sin embargo, los valores de UOsm mínima obtenidos en nuestro estudio fueron similares a los establecidos en personas adultas y niños [350 mOsm/kg] (Bockenhauer y Aitkenhead, 2011; Chan et al. 2002) y en ancianos [300 mOsm/kg] (Hebert et al. 2003).

Al evaluar la UOsm en el segundo estudio se observó, su tendencia a disminuir a medida que progresaba la enfermedad renal. De modo similar, en la leishmaniosis humana, se han observado defectos en la concentración urinaria caracterizados por una disminución de la UOsm sin alteración de la GFR y en ausencia de azotemia (Oliveira et al. 2011). En personas con CKD y azotemia también se ha asociado la hiposmolalidad urinaria persistente, con una disminución acelerada de la GFR y una progresión más rápida de la enfermedad renal (Hebert et al. 2003). Nuestros resultados podrían ser un indicio temprano del daño tubular renal secundario a CanL, y podrían indicar su evolución. Así

mismo, luego de instaurar el tratamiento se observó la tendencia a aumentar de la UOsm. Cabe señalar que en el grupo de seguimiento, todos los perros presentaban proteinuria, pero menos de la tercera parte presentaban azotemia (datos no presentados). Es probable que no se haya detectado una relación significativa entre la proteinuria y la UOsm, debido a que la proteinuria en la CanL es predominantemente mixta o glomerular (Zatelli et al. 2003; Ibba et al. 2016), por lo cual la UOsm podría subestimar los cambios asociados a la eficacia del tratamiento a nivel de la funcionalidad glomerular. Sin embargo, nuestros resultados podrían indicar la mejoría de la función renal en general, como respuesta al tratamiento.

Así mismo, en el primer estudio hemos tenido en cuenta el efecto de la edad para establecer los intervalos de referencia, ya que al igual que se describe en la literatura (van Vonderen et al. 1997; Sands et al. 2012; Gekle et al. 2017), hemos observado que la UOsm disminuye a medida que los perros envejecen. La disminución en la capacidad para concentrar y diluir la orina a medida que el sistema renal envejece se ha asociado con varias alteraciones funcionales intrínsencas, tales como la reducción de la GFR, la disminución en la reabsorción de Na, la excreción de K, la síntesis de vitamina D3, la excreción neta de ácidos; así mismo, en la respuesta a hormonas como la ADH, en la actividad del sistema renina-angiotensina-aldosterona, la resistencia parcial al efecto del péptido natriurético atrial y la disminución en la capacidad de autoregulación renal (van Vonderen et al. 1997; Gekle et al. 2017); y a causas extrínsecas como el aumento de la resistencia vascular renal (Gekle et al. 2017).

En cuanto al efecto del sexo sobre la UOsm, los resultados del primer estudio no indican que exista una diferencia significativa entre machos y hembras, aunque se ha descrito en estudios previos en perros (Izzat et

al. 1989) y en humanos (Perucca et al. 2007; Perinpam et al. 2016). Sin embargo, al analizar el efecto del estado reproductivo sobre la concentración urinaria, la UOsm en hembras esterilizadas resultó menor que en las hembras intactas. En el primer estudio no encontramos una explicación para estos resultados, pero la respuesta podría atribuirse al efecto de los estrógenos sobre el sistema renal.

Recientemente, en las mujeres se ha descrito el efecto de los estrógenos en la ralentización del envejecimiento renal (Baylis, 2012). Los estrógenos ejercen diversos efectos positivos al inhibir el desarrollo de la glomeruloesclerosis promoviendo la reparación vascular y protegiendo los podocitos del epitelio renal contra la apoptosis, así como evitando la hipertensión dependiente del envejecimiento. Es probable que, al enlentecer el proceso de envejecimiento renal, indirectamente la acción de los estrógenos, ayude a mantener la capacidad de concentrar la orina en las mujeres antes de la menopausia (Baylis, 2012). Podría plantearse la hipótesis de que en las perras esterilizadas suceda un fenómeno similar, y que su sistema renal no sea influenciado por los efectos beneficiosos de los estrógenos, de modo que se acelere el proceso de envejecimiento renal y la disminución en la concentración urinaria se manifieste en una edad más temprana. Sin embargo, se requieren estudios específicos en perros para determinar el efecto de los estrógenos en el sistema renal y la UOsm en hembras esterilizadas.

Al igual que la UOsm, las FEs también se han descrito como biomarcadores útiles para evaluar la funcionalidad tubular renal, siendo capaces de predecir la tendencia en las alteraciones electrolíticas y la evolución de la AKI y CKD en perros (Lefebvre et al. 2008; Brown et al. 2015).

Últimamente, el Mg ha cobrado importancia en el estudio de la enfermedad renal ya que se ha demostrado que los desequilibrios de este electrolito son frecuentes en la AKI y CKD. Normalmente, el valor del magnesio sérico depende casi en su totalidad de la correcta funcionalidad renal y de la adecuada reabsorción del Mg en el segmento ascendente grueso del asa de Henle, los túbulos proximales y los túbulos contorneados distales (Feinsfeld et al. 2015). La hipomagnesemia se ha relacionado con un aumento del riesgo de mortalidad en personas con AKI, en tratamiento con hemodiálisis, y cómo un factor de riesgo para el desarrollo de CKD y de enfermedad renal terminal (Feinsfeld et al. 2015; Li et al. 2015).

En el segundo estudio, las FEs de Na, Cl y Mg incrementaron con la progresión de la enfermedad renal. Estos resultados concuerdan con datos previos que describen aumento de la FENa sin azotemia en perros con leishmaniosis, probablemente debido a daño glomerular (Sousa et al. 2016). Del mismo modo, se ha observado el aumento de la FENa y FECl en perros con CKD (Lefrebvre et al. 2008), y de la FEMg en personas con CKD para mantener los niveles séricos de dicho electrolito (Feinsfeld et al. 2015). Además, nuestros resultados se relacionan en parte con estudios previos en humanos, en los que se ha observado aumento de la FENa y FEMg en presencia de hiponatremia e hipomagnesemia, asociadas con disfunción tubular renal asintomática secundaria a leishmaniosis (Lima Verde et al. 2007; Oliveira et al, 2011; Silva Junior et al. 2014). Sin embargo, en nuestra investigación no se observó una disminución significativa de los niveles séricos de ninguno de los electrolitos evaluados (datos no presentados) en relación con el aumento de la FE de los mismos.

Así mismo, en el segundo estudio se observó que los valores de FEMg aumentaron de modo más significativo en los perros en riesgo de desarrollar la enfermedad renal (no proteinúricos y no azotémicos), que en aquellos con una enfermedad renal evidente (proteinuria y azotemia). Además, se observó la mejoría de dichos valores con el tratamiento. Nuestros resultados concuerdan con los estudios en humanos que han demostrado que la FEMg es un indicador sensible para detectar de forma precoz anormalidades estructurales y funcionales a nivel tubulointersticial (Futrakul et al. 1999; Deekajorndech, 2009) antes de la presentación de azotemia en los estadios tempranos de la CKD (Gheissari et al. 2011; Noiri et al. 2015).

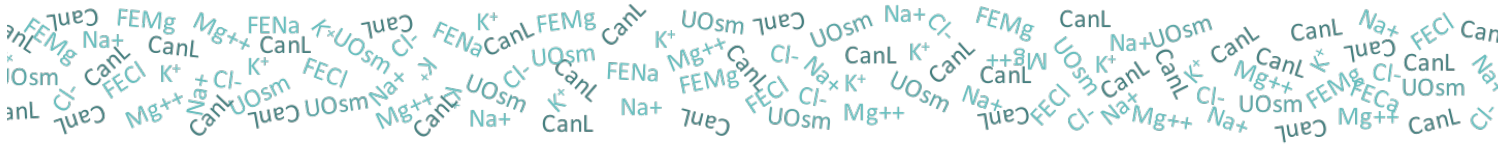
Con base en nuestros resultados puede afirmarse que la FEMg es un biomarcador útil para detectar de forma temprana el daño renal inicial en perros con leishmaniosis, y que su evaluación podría ser útil para evaluar la respuesta al tratamiento.

La FEK tuvo tendencia a aumentar a medida que progresó la enfermedad renal, pero no de forma significativa, al igual que se ha observado en humanos en diferentes estadios de la CKD. El mantenimiento en los valores de la FEK podría ser el resultado de la activación de mecanismos compensatorios que favorecen la conservación de Na, y de forma secundaria la del K a nivel tubular (Ueda et al. 2016).

Existen varias limitaciones en el segundo estudio de la investigación. Una de ellas fue la *n* reducida de la población, que pudo ser la causa de la falta de significancia en algunos de los resultados, y limitó el empleo de los intervalos de referencia de la UOsm en el mismo. Sin embargo, es probable que el efecto de la edad en dicho estudio fuera

insignificante, ya que la mayoría de animales eran adultos (datos no presentados).

Además, en el segundo estudio no se incluyó un grupo de animales control, por lo tanto, no fue posible comprobar si los analitos estudiados podrían ser útiles para diferenciar animales enfermos, de animales sanos. Se necesitarán estudios en una población extensa, con un grupo control y un seguimiento del tratamiento más prolongado, para determinar la utilidad de la UOsm en la detección temprana y en la evaluación del daño renal en la CanL.



10. Conclusiones

1. La medición de la osmolalidad urinaria mediante la osmometría de punto de congelación es un método preciso, eficaz, económico y fácil de emplear, siendo adecuado para su implementación en el ámbito clínico.

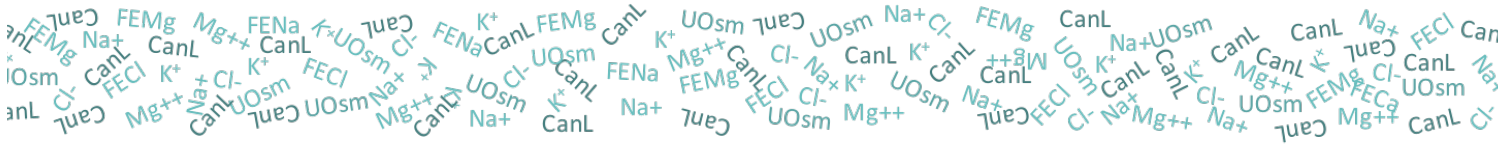
2. Se recomienda el uso de diferentes intervalos de referencia en perros jóvenes, adultos y ancianos, para la interpretación clínica de la osmolalidad urinaria.

3. La concentración de la orina evaluada mediante la osmolalidad urinaria disminuye en perros ancianos sanos. El sexo no afecta la concentración urinaria en perros sanos, pero la osmolalidad urinaria disminuye con la esterilización en las hembras.

4. La osmolalidad urinaria tiene tendencia a disminuir a medida que progresa la enfermedad renal en perros con leishmaniosis.

5. El aumento progresivo de las fracciones de excreción de sodio, cloro y magnesio, se encuentran relacionado a la presentación de azotemia y la severidad de la enfermedad renal secundaria a leishmaniosis canina.

6. La reducción de la fracción de excreción del magnesio después de un periodo corto de tratamiento no se encuentra asociado a la mejoría de la proteinuria y es un indicador de recuperación de la funcionalidad tubular en la enfermedad renal secundaria a leishmaniosis canina. Su medición podría ser útil para monitorizar el tratamiento.



11. Conclusions

1. Urine osmolality measurement by freezing point depression osmometry is a precise, accurate, inexpensive and easy to use method, making it suitable for clinical setting.

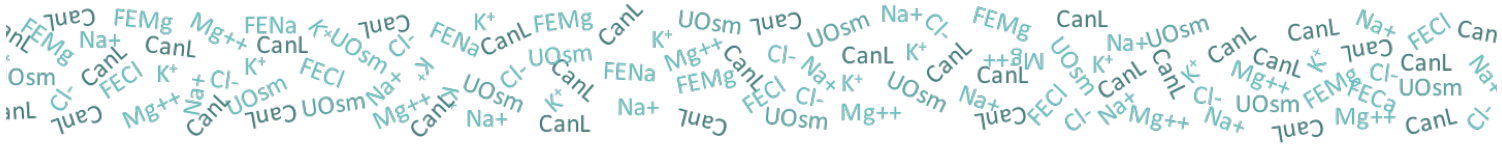
2. Different reference intervals should be used in young, adult and senior dogs for the clinical interpretation of urine osmololality.

3. Urine concentration evaluated by urine osmolality decrease in healthy elderly dogs. Sex has no effect in urine concentration values in healthy dogs, but urine osmolality decreased with sterilization in female dogs.

4. Urine osmolality tends to decrease as the renal disease progress in dogs with leishmaniosis.

5. Increase of fractional excretion of sodium, chlorine and magnesium is associated with azotemia presentation and the severity of the renal disease secondary to canine leishmaniosis.

6. Reduction in fractional excretion of magnesium after a short period of treatment has no correlation with proteinuria improvement and indicates recovery of the tubular function in renal disease secondary to canine leishmaniosis. Its measurement could be helpful to monitor the treatment.



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