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Novel insights into canine proteinuria

Ph.D. Dissertation

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1. List of abbreviations

ACEi	Angiotensin-converting enzyme inhibitor
AER	Albumin excretion rate
Ang II	Angiotensin II
ARB	Angitensin receptor blocker
CI	Confidence interval
CKD	Chronic kidney disease
DM	Diabetes mellitus
PDH	Pituitary-dependent hyperadrenocorticism
ELISA	Enzyme-linked immunoassay
ERD test	Early renal disease screening test
EM	Electron microscopy
FSGS	Focal segmental glomerulosclerosis
GFB	Glomerular filtration barrier
GFR	Glomerular filtration rate
ICGN	Immune complex-mediated glomerulonephritis
IRIS	International Renal Interest Society
LM	Light microscopy
PCR	Polymerase chain reaction
PLN	Protein-losing nephropathy
RL-NOS	Renal lesions not otherwise specified
UAC	Urinary albumin-to-creatinin ratio
UAlb	Urinary albumin concentration
UPC	Urinary protein-to-creatinin ratio
PUFA	Polyunsaturated fatty acids
RAAS	Renin-angiotensin-aldosterone system
RI	Reference interval
SAA	Serum amyloid A
SCWT	Soft coated wheaten terrier
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSA	Sulfosalicylic acid

2. Summary

Albuminuria and proteinuria are both hallmarks of glomerular dysfunction. As albuminuria often precedes proteinuria, its measurement can be helpful in the early recognition of kidney diseases. Albuminuria has been detected in dogs with several inflammatory, infectious, and neoplastic diseases. It has long been known that *Dirofilaria immitis* (heartworm) can cause proteinuria and albuminuria as consequences of glomerular damage. Many studies described membranoproliferative glomerulonephritis in dogs with spontaneous and experimentally induced heartworm infection. Although glomerular lesions can be pronounced, kidney failure and azotemia are usually absent. In Europe, *Dirofilaria repens,* and *D. immitis* are both dispersed. *D. repens* causes subcutaneous and ocular dirofilariasis in canids and is considered a less harmful infection than heartworm. It is unknown whether *D. repens* is capable of causing similar glomerular lesions to those caused by *D. immitis*.

Thus far, no species-specific reference interval (RI) for albuminuria has been established for dogs – previous studies examining albuminuria in dogs and cats compared small healthy control groups with the various diseased groups.

Our first study aimed to establish an RI for albuminuria in more than 120 healthy dogs that is the recommended limit of reference individuals to determine reliable reference limits. Our second goal was to define breed-specific reference intervals in case we found significant differences between the albuminuria of different breeds. Sighthounds were a point of interest because they are known to have unique reference values, and Greyhounds were previously shown to excrete more albumin than other breeds. Breed-specific reference intervals for Beagle dogs could be helpful because of their frequent use as laboratory animals.

Our second study aimed to investigate whether *D. repens* is capable of causing similar glomerular dysfunction as heartworm infection and, thus, whether *D. repens* infected dogs have a higher magnitude of proteinuria or albuminuria than non-infected dogs kept under the same circumstances. Our study also aimed to explore whether the magnitude of proteinuria or albuminuria would decrease after topical moxidectin treatment. In addition to our study's primary and secondary goals, we compared some other laboratory variables (hematology, serum urea, creatinine, urine specific gravity) between the infected and non-infected dogs.

One hundred sixty-four clinically healthy dogs were enrolled in the first study. Urinary albumin was determined by the immunoturbidimetric method, and albumin excretion was expressed as the urinary albumin-to-creatinine (UAC) ratio. After exclusions, the reference population comprised one hundred twenty-four clinically healthy dogs of thirty-two breeds. The median UAC of the study population was 3.0 mg/g (range: 0 – 48 mg/g).

This work is the first to establish an RI for UAC in dogs. The RI was defined as 0 – 19 mg/g (with a 90% confidence interval for the upper limit of 13 – 28 mg/g). No significant difference was found between male and female dogs or between different age and body weight groups. The results of Sighthounds (n=30) and Beagle dogs (n=23) did not differ from the other breeds. The method of collection (cystocentesis or free-catch) did not seem to influence the UAC values.

In the second phase of our research, sixty-five clinically healthy laboratory beagle dogs were enrolled in a cross-sectional study. Dogs were tested for *D. repens* infection (modified Knott test, PCR test, *D. immitis* antigen test) and grouped as "*D. repens* infected" or "control" dogs. UAC and urinary protein-to-creatinine ratio (UPC) were measured from samples taken by cystocentesis. Forty-three (26 infected, 17 control) dogs were included in the final study group.

UAC but not UPC level was significantly higher in the infected group (UAC median 12.5; range, 0 – 700 mg/g and UPC median 0.15; range, 0.06 –1.06) than in the control group (UAC median 6.3; range, 0 – 28 mg/g and UPC median 0.13; range, 0.05 – 0.64; p=0.02 and p=0.65). Albuminuria (UAC >19 mg/g) was detected in 9/26 (35%) dogs in the infected group and in 2/17 dogs (12%) in the control group. Overt proteinuria (UPC >0.5) was present in 6/26 (23%) of the infected dogs and in 1/17 (6%) of the control dogs.

Eosinophil granulocyte cell count was higher in the infected group (0.79 ± 0.36 G/L) than in control (0.45 ± 0.29 G/L), while eosinophilia was present in 10/26 (39%) dogs in the infected group. Thrombocyte count was also higher in the infected group (439.4 ± 165.1 G/L) than in control (355.6 ± 84.9 G/L), while thrombocytosis was present in 11/26 (42%) dogs in the infected group. No difference was detected in the serum urea or creatinine values, nor the urine specific gravity results. One or two topical moxidectin treatments did not change the UAC and UPC values.

We concluded in the first study that the human RI of UAC is somewhat higher (0 – 30 mg/g) than the canine (0 – 19 mg/g), but it is very similar to the upper limit 90% confidence interval of canine UAC (13 – 28 mg/g), found in this study.

We concluded in the second study that as glomerular diseases are often present in non-azotaemic dogs with acceptable urine-concentrating ability, the presence of albuminuria raises the suspicion of early glomerular dysfunction caused by *D. repens* infection.

3. Összefoglaló

Újabb ismeretek kutyák proteinuriájáról

Mind az albuminuria, mind a proteinuria a glomerulopathiák fő jellemzői. Mivel az albuminuria gyakran megelőzi a proteinuria kialakulását, mérése hasznos lehet a vesebetegségek korai felismerésében. Albuminuria megjelenését számos gyulladásos, fertőző és daganatos kórképekben igazolták. Régóta ismert, hogy a *Dirofilaria immitis* okozta szívférgesség glomeruluskárosodáshoz vezet, így gyakori következmény az albuminuria és proteinuria megjelenése. Számos tanulmányban számoltak be membranoproliferatív glomerulonephritis kialakulásáról mind spontán, mind pedig mesterségesen előidézett szívféreg fertőzöttség esetén. Bár a glomeruluskárosodás kifejezett lehet, azotaemia és veseelégtelenség ritkán jellemzi a kórképet. Európában, a szívféreg mellett, annak közeli rokona, a bőrféreg (*Dirofilaria repens*) is elterjedt. A *D. repens* a kutyafélék bőr- és szemférgességét okozza, és a szívférgességnél kevésbé ártalmas fertőzésnek tekinthető. Arról nincsenek ismereteink, hogy a bőrférgesség, a szívférgességhez hasonlóan, okoz-e glomeruluskárosodást.

Mostanáig nem állapítottak meg fajspecifikus referenciatartományt kutyák albuminuriájára. Azokban a kutatásokban, amelyekben kutyák és macskák albuminuriáját vizsgálták, kislétszámú kontrollcsoportok eredményeit hasonlították össze a különböző beteg csoportokéval.

Első vizsgálatunk célja az albuminuria referenciatartományának meghatározása volt, több, mint 120 egészséges kutya bevonásával. Ekkora referenciapopuláció szükséges egy megbízható referenciaérték megállapításához. További célunk volt fajtaspecifikus referenciatartományok meghatározása abban az esetben, ha szignifikáns különbséget találunk a különböző fajták albuminuriája között. Minél több agár, illetve beagle fajtájú kutyát terveztünk bevonni a vizsgálatba. Az agarakról ismert, hogy egyedi laboratóriumi referenciaértékekkel rendelkeznek, valamint, egy korábbi kutatásban az agarak nagyobb mértékben ürítettek albumint, mint fajtársaik. Beagle kutyák fajtaspecifikus referenciaintervallumai hasznosak lehetnek gyakori, kísérleti állatként történő felhasználásuk miatt.

Második kutatásunk célja annak vizsgálata volt, hogy jelentkezik-e a *D. repens*-szel fertőzött kutyáknál proteinuria vagy albuminuria, vagyis, a szívférgességhez hasonlóan, okoz-e a bőrférgesség is glomeruluskárosodást. Vizsgálatunk célja volt annak feltárása is, hogy a proteinuria vagy az albuminuria mértéke változik-e moxidektin spot-on kezelés

hatására. Vizsgálatunk elsődleges és másodlagos céljai mellett néhány további laboratóriumi paramétert (hematológia, szérum karbamid, kreatinin, vizelet sűrűség) is összehasonlítottunk a fertőzött és nem fertőzött kutyák között.

Százhatvannégy klinikailag egészséges kutyát vontunk be az első vizsgálatba. A vizelet albuminkoncentrációját immunoturbidimetriás módszerrel határoztuk meg, majd vizelet albumin/kreatinin (UAC) arányként fejeztük ki. A kizárások után a referenciapopuláció százhuszonnégy klinikailag egészséges kutyából állt, melyek harminckét fajtához tartoztak. A vizsgált populációban a medián UAC 3,0 mg/g volt (tartomány: 0 – 48 mg/g).

A jelen munkában elsőként határoztuk meg kutyák UAC értékének referenciatartományát. A referenciatartományt 0 – 19 mg/g között állapítottuk meg (a konfidencia intervallum felső 90%-os határa pedig 13 – 28 mg/g volt). Nem találtunk szignifikáns különbséget a hím és nőstény ivarú kutyák, illetve a különböző kor- és testsúlycsoportok között. Az agarak (n=30) és a beagle kutyák (n=23) eredményei nem különböztek a többi fajtáétól. A mintavétel módja (cystocentesis vagy spontán ürített) nem befolyásolta az UAC értékeket.

Kutatásunk második szakaszában, egy keresztmetszeti klinikai vizsgálatba, hatvanöt klinikailag egészséges laboratóriumi beagle kutyát vontunk be. A kutyákat szűrtük *D. repens* fertőzésre (módosított Knott-teszt, PCR-teszt, *D. immitis* antigénteszt), majd az eredmények alapján „*D. repens*-fertőzött" vagy „kontroll" csoportokba soroltuk. Az UAC-t és a vizelet fehérje/kreatinin arányát (UPC) cystocentesissel vett vizeletmintákból határoztuk meg. Kizárások után negyvenhárom (26 fertőzött, 17 kontroll) kutyát vizsgáltunk.

Az UAC szignifikánsan magasabb volt a fertőzött csoportban (medián 12,5; tartomány 0 – 700 mg/g), mint a kontrollcsoportban (medián 6,3; tartomány 0 – 28 mg/g) (p=0,02). Az UPC értékek nem különböztek szignifikánsan a fertőzött (medián 0.15; tartomány 0.06 – 1.06) és a kontroll csoport (medián 0,13; tartomány 0,05 – 0,64) között (p=0,65). Albuminuriát (UAC >19 mg/g) a fertőzött csoportban 9/26 (35%), a kontrollcsoportban pedig 2/17 kutyánál (12%) mutattunk ki. Jelentős proteinuriát (UPC >0,5) a fertőzött kutyák 23%-ánál (6/26), valamint a kontroll kutyák 6%-ánál (1/17) tapasztaltunk.

Az eozinofil granulocitaszám magasabb volt a fertőzött csoportban (0,79 ± 0,36 G/L), mint a kontrollcsoportban (0,45 ± 0,29 G/L). Eosinophiliát találtunk a fertőzött kutyák 39%-ánál. A thrombocytaszám szintén magasabb volt a fertőzött csoportban (439,4 ± 165,1 G/L), mint a kontrollcsoportban (355,6 ± 84,9 G/L), míg thrombocytosis a fertőzött kutyák 42%-ánál jelentkezett. Nem volt különbség a szérum karbamid- vagy kreatinin tartalmában, sem a vizelet sűrűségében a két csoport között. Egy, vagy két moxidektin spot-on kezelés nem változtatta meg az UAC és UPC értékeket.

Az első vizsgálatban arra a következtetésre jutottunk, hogy a humán vizelet albumin/kreatinin arány referenciatartománya valamivel magasabb (0 – 30 mg/g), mint a kutyáké (0 – 19 mg/g), de nagyon hasonló a kutya referenciaérték felső határának 90%-os konfidencia intervallumához (13 – 28 mg/g), melyet ebben a tanulmányban találtunk.

A második vizsgálatban arra a következtetésre jutottunk, hogy mivel a glomeruláris megbetegedések gyakran fordulnak elő elfogadható vizeletkoncentráló képességgel rendelkező, nem azotémiás kutyákban, az albuminuria jelenléte felveti a *D. repens* fertőzés által okozott korai glomeruláris károsodás gyanúját.

4. Introduction and objectives

Detecting proteins in the urine has a long history. Around 400 B.C. Hippocrates observed that "when bubbles settle on the surface of the urine, it indicates disease of the kidneys and that the complaint will be protracted." In the 17th century, urinary proteins were found and described as "coagulating material." In the early 18th century, a Swiss doctor described the symptoms of nephrotic syndrome, but it was only linked to kidney disease and proteinuria in the early 19th century. A review suggests that Mozart's death in 1791 was related to proteinuric kidney disease, and he died after developing severe edemas. The appearance of simple tests for detecting urinary proteins in the mid-19th century was a cornerstone in diagnosing proteinuric diseases (Cattran, 2011).

A small amount of protein in the urine of cats and dogs is physiologic. Proteinuria means an abnormal extent of protein loss in the urine. Persistent proteinuria is defined as an abnormal magnitude of proteins in the urine detected on three or more occasions, two or more weeks apart (Lees et al., 2005). Persistent proteinuria found with an inactive urine sediment is generally consistent with renal damage (Harley & Langston, 2012). Proteinuria has high clinical importance. Many studies proved that proteinuria is associated with shorter survival time, not only in people but in cats and dogs as well (Jacob et al., 2005; Syme et al., 2006; Jepson et al., 2007; Chakrabarti et al., 2012). In people, the magnitude of protein loss in the urine is associated with the progression of chronic kidney disease (CKD), and it is a prognostic factor in diabetic nephropathy and some cardiac diseases as well (Harley & Langston, 2012). Medical reduction of proteinuria prolongs the survival of people, dogs, and cats (Lees et al., 2005; Littman, 2011).

Albuminuria means abnormal loss of albumin in the urine. In people, albuminuria is considered the most important marker of renal damage and an essential predictor of CKD progression into dialysis-dependent kidney failure (KDIGO, 2013; Murton et al., 2021). Apart from kidney diseases, urinary albumin excretion is a marker of generalized vascular dysfunction and a risk marker of cardiovascular events (e.g., heart attack, stroke) (Gerstein et al., 2001). In human medicine, albuminuria is a negative prognostic factor; its presence and magnitude are associated with increased cardiovascular and also all-cause mortality (Hillege et al., 2002).

In veterinary medicine, many studies found that albuminuria can precede proteinuria and thus can help in the early diagnosis of renal damage (Vaden et al., 2011, Lees et al., 2002; Grauer et al., 2002). Albuminuria negatively correlates with survival in cats with CKD (Syme et al., 2006) and critically ill dogs (Vaden et al., 2010). The measurement of albuminuria is

suggested as a screening test for early renal damage in dogs predisposed or suspected to have renal disease (e.g., hereditary nephropathies) and who have hypertension or systemic diseases leading to proteinuria (Harley & Langston, 2012). Although the importance of albuminuria has become apparent recently, the reference interval for albuminuria in dogs has not yet been established. Because of this, previous studies examining albuminuria in different conditions compared urinary albumin values of the diseased animals to healthy control groups (Mazzi et al., 2008; Schellenberg et al., 2008; Bacic et al., 2010; Smets et al. 2010; Schaefer et al., 2011). These control groups consisted of 10-40 individuals. To define a reliable reference limit, at least 120 individuals are required (Friedrichs, 2012).

In dogs, albuminuria has already been detected in many different illnesses: familial glomerulopathies (Vaden et al., 2001; Lees et al., 2002), leishmaniasis (Cortadellas et al., 2008), diabetes mellitus (Herring et al., 2014; Mazzi et al., 2008), pituitary-dependent hyperadrenocorticism (Mazzi et al., 2008), CKD (Smets et al., 2010), hypertension (Bacic et al., 2010), critical illnesses (Vaden et al., 2010; Whittemore et al., 2011), systemic inflammatory response syndrome (Schaefer et al., 2011), lymphoma, and osteosarcoma (Pressler et al., 2003). Many studies described membranoproliferative glomerulonephritis in dogs with spontaneous and experimentally induced *Dirofilaria immitis* infection (Casey et al., 1975; Grauer et al., 1989; Paes-de-Almeida et al., 2003). Albuminuria and proteinuria are commonly associated with heartworm disease (Grauer et al., 2002; Morchón et al., 2012; Hormaeche et al., 2014). In Hungary, the prevalence of *Dirofilaria repens* is higher than *D. immitis* (Farkas et al., 2020). No studies have examined the possible glomerular damage caused by *D.repens*.

Our first study aimed to establish a reference interval for albuminuria in more than 120 healthy dogs, as this is the minimum recommended number for reliable results. Our second goal was to define breed-specific reference intervals in case we found significant differences between the albuminuria of different breeds. Sighthounds were a point of interest because these dogs are known to have unique reference values (Zaldívar-López et al., 2011) and Greyhounds were previously shown to excrete more albumin than other breeds (Surman et al., 2012). Breed-specific reference intervals for Beagle dogs could be helpful because of their frequent use as laboratory animals.

Our second study aimed to investigate whether *D. repens* is capable of causing similar glomerular lesions as heartworm infection and, thus, whether *D. repens* infected dogs have a higher magnitude of proteinuria or albuminuria than non-infected dogs kept under the same circumstances. Our study also aimed to explore whether the magnitude of proteinuria or albuminuria would decrease after topical moxidectin treatment.

In addition to our study's primary and secondary goals, we compared some other laboratory variables (hematology, serum urea, creatinine, urine specific gravity) between the infected and non-infected dogs.

5. Literature review of proteinuria

5.1 Pathophysiology of proteinuria

The healthy glomerulus is an "elegant sieve", filtering about 20% of the cardiac output. It produces liters of ultrafiltrate per day. Water and small molecules, including smaller-sized and positively charged proteins, can freely pass through the glomerular filtration barrier, while bigger-sized (>69 kDa) and negatively charged proteins, like albumin, are withheld (Littman, 2011).

The glomerular capillary wall has three layers: the fenestrated endothelium, the glomerular basement membrane, and the podocytes (see Figure 1).

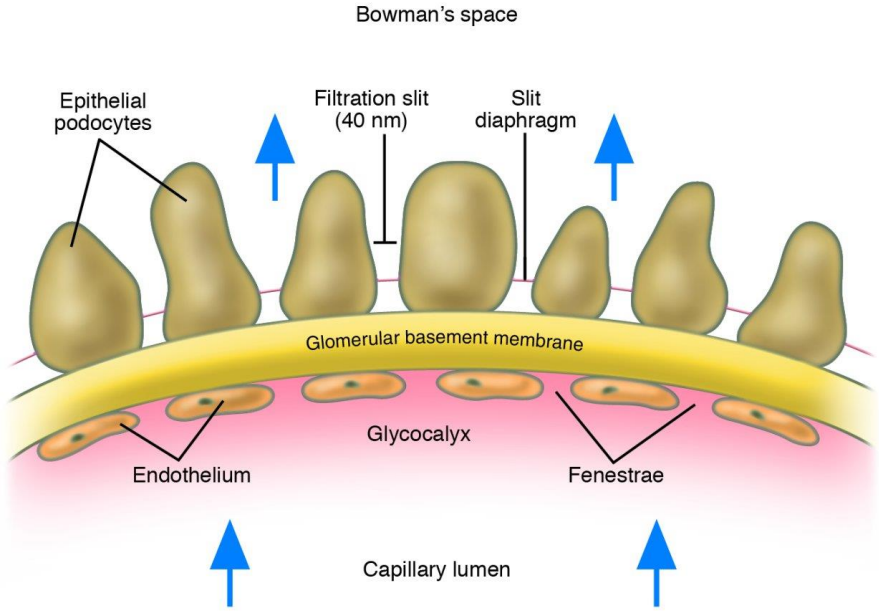

Figure 1. Structure of the glomerular capillary wall (Deen et al., 2004)

If any part of the wall gets injured, proteinuria of glomerular origin will occur. There are relatively large pores on the endothelium that are bigger in size than albumin. The endothelium is covered by a negatively charged glycocalyx surface that helps to keep negatively charged proteins in the circulation. The glomerular basement membrane contains many proteins (e.g., collagen and laminin), forming a network of fibrils. These proteins are highly hydrated, resulting in a gel-like structure. Under these layers, the foot processes of the podocytes form the most critical anatomical barrier for proteins. A porous intercellular junction, the slit diaphragm connects neighbouring foot processes (Littman, 2011; Sharma &

Smyth, 2021). The few proteins that can pass the glomerular filtration barrier are almost completely reabsorbed by the proximal tubular cells. This reabsorption is driven by two receptors, megalin and cubilin, which bind the proteins from the ultrafiltrate. The receptor-protein complexes are endocytosed, and proteins are degraded in lysosomes. Proteins are hydrolyzed, and amino acids are returned to the plasma (Harley & Langston, 2012; Sharma & Smyth, 2021). This process can be saturated. Tubular proteinuria will occur if this transport maximum is reached (Grauer, 2011).

Proteinuria itself can accelerate the progression of kidney diseases. When the aforementioned tubular intracellular pathways are overwhelmed and are not able to handle excess albumin, tubular cells start to produce proinflammatory (e.g., cytokines) and profibrotic mediators (e.g., transforming growth factor-ß1). Exposure to albumin causes other changes in tubular cell function that lead to apoptosis of tubular cells (Sharma and Smyth, 2021).

5.2 Classification of proteinuria

Proteinuria can be physiologic or pathologic. Physiologic (functional) proteinuria is usually mild and transient, e.g., after strenuous exercise, seizures, a febrile episode, exposure to extreme weather conditions, or stress (Grauer, 2011).

Pathologic proteinuria can be divided into three categories based on etiology: pre-renal, renal or post-renal. Pre-renal proteinuria is "due to abnormal plasma content of proteins that traverse glomerular capillary walls having normal permselectivity properties" (Lees et al., 2005). It occurs when an excessive amount of circulating low molecular weight proteins (e.g., myoglobin, hemoglobin, Bence-Jones proteins) overwhelm the transport capacity of the proximal tubular cells. Thus, proteins appear in the urine. Systemic hypertension can also cause pre-renal proteinuria as increased hydrostatic pressure on the glomerular filtration barrier can expand the size of the pores on the endothelial layer (Harley & Langston, 2012; Lees et al., 2005).

Pathologic renal proteinuria is "abnormal renal handling of normal plasma proteins and a proteinuria that is attributable to structural or functional lesions within the kidneys" (Lees et al., 2005). Table 1 lists the different etiologies of pathologic proteinuria.

Renal proteinuria can be categorized by the localisation of the lesions: glomerular, tubular, or interstitial. Glomerular proteinuria means increased permeability of the glomerular filtration barrier because of glomerular lesions. Tubular lesions and impaired reabsorption of proteins in the proximal tubules cause tubular proteinuria. Interstitial proteinuria is due to an

inflammatory process of the renal interstitium (e.g., acute nephritis, leptospirosis, pyelonephritis, renal neoplasia, or nephroliths) (Harley & Langston, 2012; Lees et al., 2005).

Post-renal proteinuria happens "due to protein entry into the urine after it enters the renal pelvis" (Lees et al., 2005). It can further be divided into urinary and extraurinary origin. Post-renal proteinuria from urinary origin means hemorrhagic or exsudative processes affecting the renal pelvis, the ureter, the urinary bladder, or the urethra. Extraurinary origin means secretions, hemorrhagic or exudative processes affecting the genital tract or the external genitalia (Harley & Langston, 2012; Lees et al., 2005).

Table 1. Causes of pathologic proteinuria in dogs and cats. (Harley and Langston, 2012; Littman, 2011; Vaden, 2011)

Prerenal	**Renal**	**Postrenal**
Multiple myeloma	Acute Kidney Injury	Diseases of the lower urinary tract
Hemoglobinuria, myoglobinuria	Chronic kidney disease	Diseases of the genital tract
Systemic hypertension	Systemic hypertension	
Drug reactions	Drug reactions, corticosteroid therapy	
Acute pancreatitis	Any severe inflammatory disease (e.g. pyometra, generalized pyoderma, endocarditis, pancreatitis, peritonitis), neoplastic disease, infectious or immune-mediated disease	
Hyperthyroidism	Viral infections (FIV, FeLV, FIP; CAV-1)	
	Leptospirosis	
Hyperadrenocorticism	Vector-born diseases (Lyme disease, Dirofilariosis, Ehrlichiosis, Anaplasmosis, Babesiosis, Leishmaniasis)	
	Diabetes mellitus, Hyperthyroidism	
	Hyperadrenocorticism	

5.3 Glomerular diseases

Proteinuria is a hallmark of glomerular diseases. Glomerular diseases are dogs' most frequent causes of CKD. In cats, proteinuria of glomerular origin is less common. Glomerulopathies can have immune-mediated and non-immune-mediated origins. For the definitive diagnosis, biopsy sampling and histopathologic evaluation are needed (Vaden, 2011). Previously, the classification and nomenclature for canine glomerular disease were taken from human classification schemes based on light microscopic criteria. In 2005, the

World Small Animal Veterinary Association started the Renal Standardization Project. Their goal was "defining the discipline of nephropathology, characterizing the expression of canine glomerular disease, and establishing a prototype classification system for canine glomerular disease." In this project, they established an international infrastructure for routine diagnostic pathology that not only included light microscopy (LM) but immunofluorescent and electron microscopic (EM) imaging as well (Cianciolo et al., 2013; Cowgill & Polzin, 2013). Nephropathologists emphasize the importance of EM. In the study of Cianciolo, light microscopic evaluation led to misdiagnosis in 22 out of 89 cases compared to EM (Cianciolo et al., 2016).

In the new categorisation scheme, there are three big groups of glomerulopathies: *immune complex–mediated glomerulonephritis* (ICGN), *non-ICGN*, and "*renal lesions not otherwise specified"* (RL-NOS) (see Figure 2) (Cianciolo et al., 2018).

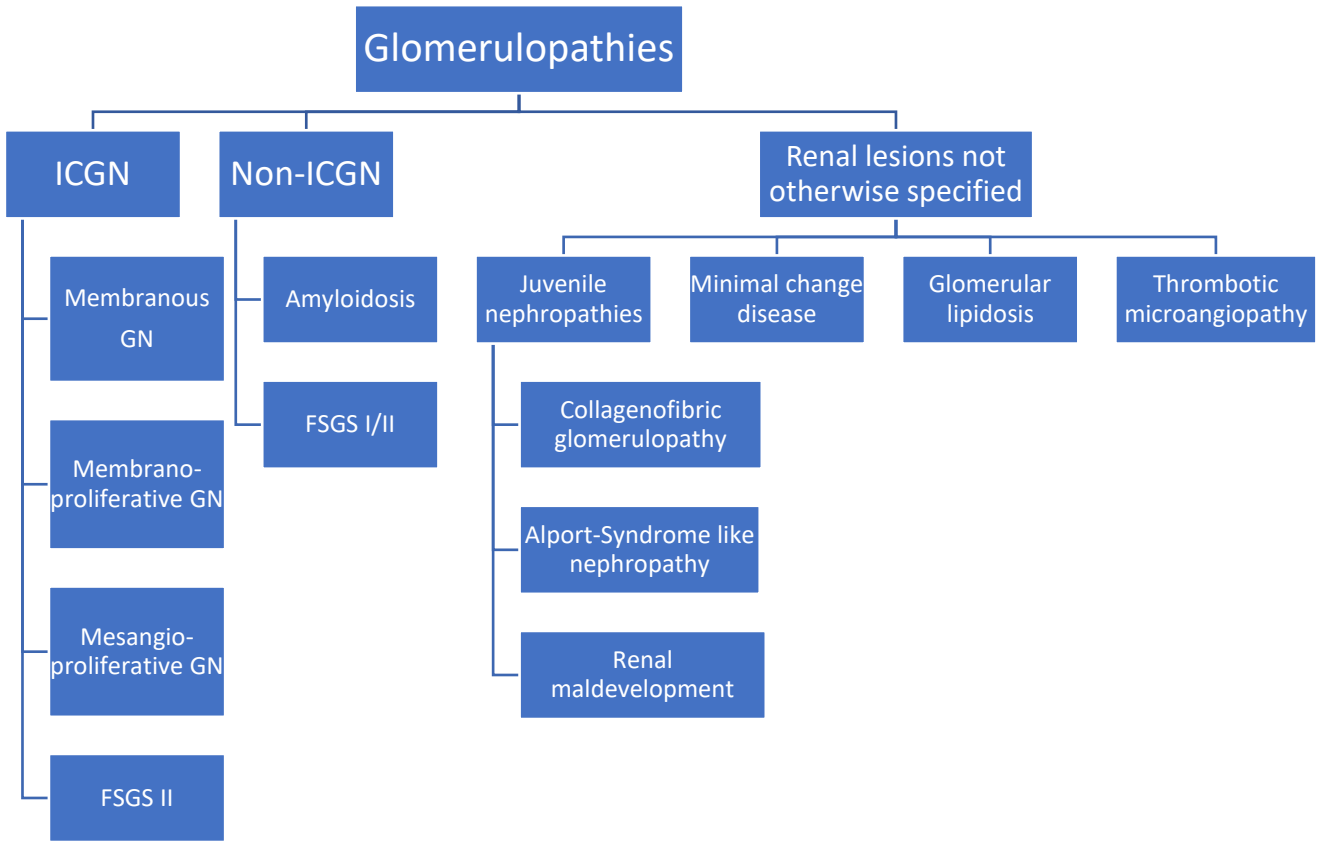

Figure 2. Histopathologic categorisation of glomerulopathies of dogs. GN= glomerulonephritis, ICGN=Immune complex-mediated glomerulonephritis, FSGS=Focal segmental glomerulosclerosis (Cianciolo et al., 2018).

The *ICGN* category includes membranoproliferative, membranous, mixed (the combination of the previous ones), and *mesangioproliferative glomerulopathies*. In a *membranous glomerulonephropathy* (Figure 3), immune complexes are deposited on the abluminal (subepithelial) side of the glomerular basement membrane, while in a *membranoproliferative glomerulonephritis*, they will be found on the luminal (subendothelial) surfaces of capillary walls. Glomeruli in *membranous glomerulonephropathy* is normocellular without inflammatory cell infiltration (that is why it is called nephropathy, not nephritis), while in *membranoproliferative GN* endocapillary hypercellularity (due to circulating leukocytes, hypertrophied endothelium, and/or mesangial cells within the capillary lumen) is typical. In *mesangioproliferative, GN* immune complexes are limited to the mesangial zone. The immune complexes lead to mesangial expansion and hypercellularity (Cianciolo et al., 2018).

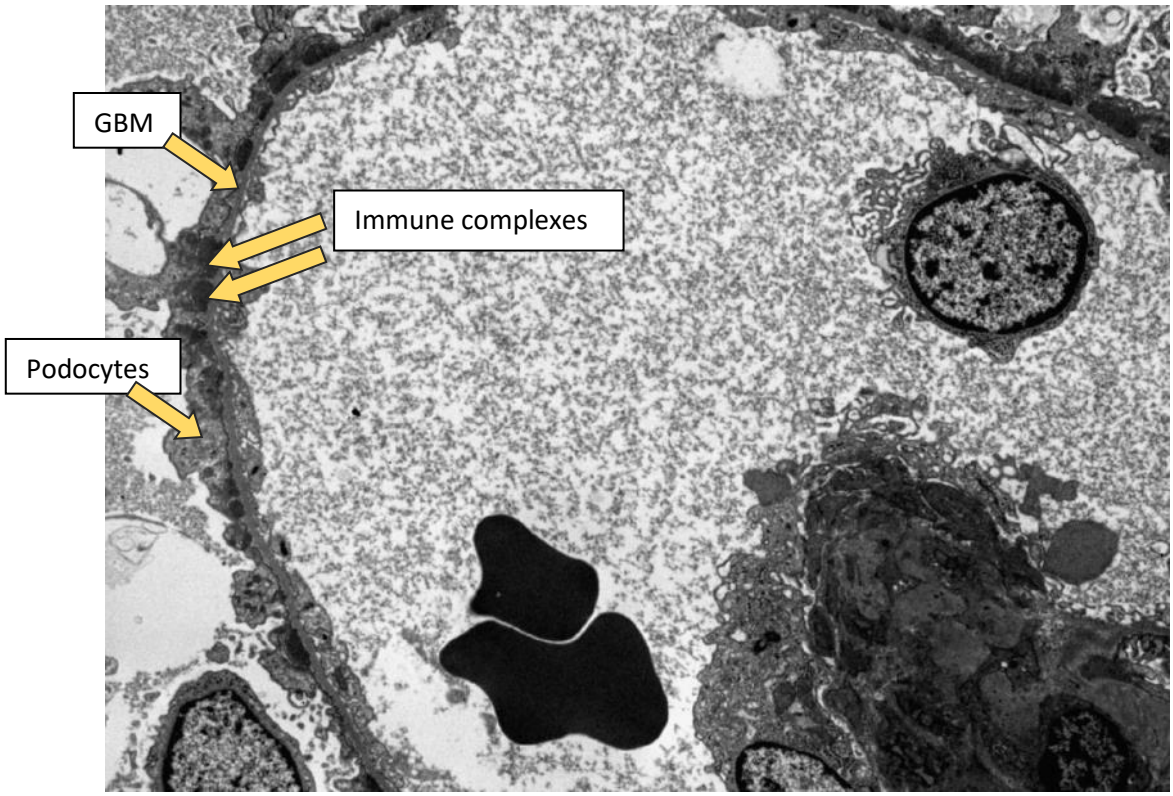

Figure 3. Transmission electron microscopic picture of a glomerulus with membranous glomerulonephropathy. "Numerous electron-dense deposits are present on the subepithelial surface of the capillary loops and there is minimal remodelling of the glomerular basement membrane (Cianciolo et al., 2018)."

The *non-ICGN* includes *focal segmental glomerulosclerosis (FSGS)* and *amyloidosis*. *FSGS* means scarring of the glomerulus with increased extracellular matrix, leading to obliteration of capillary lumens and consolidation of a portion of the glomeruli (see Figure 4). Focal means that sclerosis only involves some glomeruli (its opposite would be diffuse), and

segmental means that only a part of the glomerular tuft is involved (its opposite would be global). If the process starts with a primary injury to podocytes, it is called *FSGS I*; if the lesion occurred secondarily to any chronic glomerular disease, it is called *FSGS II*. *FSGS II* is categorised into the ICGN group if the primary glomerular injury is immune-mediated. In the disease progression, more and more glomeruli will be involved until a "*diffuse segmental to global glomerulosclerosis*" (Cianciolo et al., 2018).

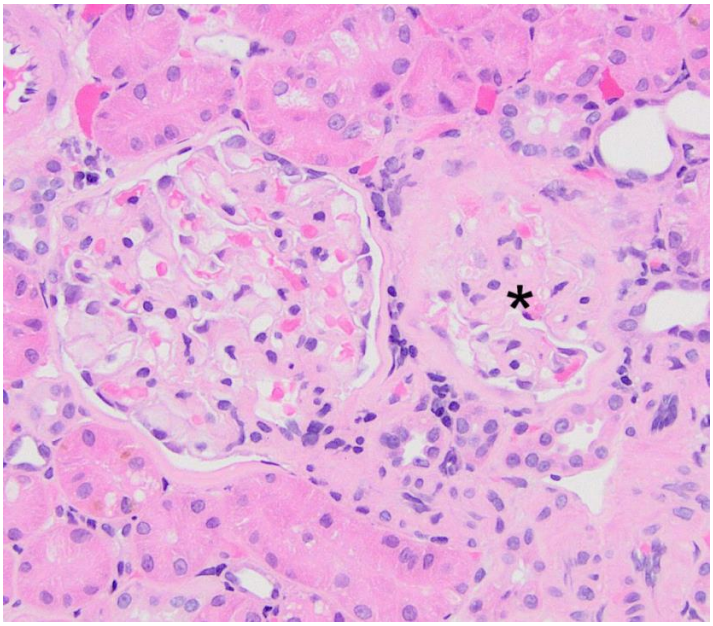

Figure 4. Light microscopic picture (hematoxylin-eosin staining) of two glomeruli. The left is normal, and the other glomerulus (asterisk) has segmental sclerosis (Cianciolo et al., 2018).

Renal amyloidosis means the deposition of extracellular insoluble proteins that consist of β-pleated structured fibrils. Histologically, nodular expansion of the mesangium and capillary walls by a congophilic material can be seen (Cianciolo et al., 2018). Amyloidosis can be inherited in Shar-Peis, beagles, English foxhounds, and Abyssinian and Siamese cats. (Littman, 2011). In people, three types of amyloidosis have been described: primary, secondary (reactive), and senile (heredofamilial).

In dogs and cats, amyloidosis is generally reactive, secondary to some inflammatory, infectious, or neoplastic processes. Amyloid deposits are formed from amyloid A protein, a fragment of the acute phase protein, serum amyloid A. Amyloid deposits can be found in the glomeruli and less frequently in the renal medulla (except in Shar-peis). DiBartola et al. examined the amyloid deposits in Shar-pei dogs. In their study, 100% of the dogs had depositions in the medulla, and 64% had glomerular depositions (DiBartola, 1990). In another study, the renal medulla was affected in 73% and the glomeruli in 79% of Shar-peis that were different from other breeds, where the medulla was only affected in 27% of the

dogs, and almost all of them (96%) had glomerular deposits (Segev, 2012). Segev et al. found that the clinical manifestation of renal amyloidosis differed between Shar-Pei and non-Shar-Pei dogs. Shar-Peis were younger, had less frequent proteinuria, hypoalbuminemia, and nephrotic syndrome, and had higher creatinine values than non-Shar-Pei dogs. In 44% of the Shar-Pei dogs, the symptoms of familial Shar-Pei fever (swollen and painful hocks and muzzle, fever, abdominal pain, gastrointestinal signs, and inappetence) preceded the renal signs (Segev et al., 2012).

The *"renal lesions not otherwise specified"* group contains *minimal change disease*, *juvenile nephropathies*, *glomerular lipidosis*, and *thrombotic microangiopathy*.

Minimal change disease is a rare, acquired podocyte injury that can be reversible. In children, it is immune-mediated and steroid-responsive. In dogs, drug-induced (masitinib) and idiopathic forms are known. On LM, the glomeruli can even look physiologic, while on EM, diffuse and global podocyte foot process effacement is seen. It typically causes overt proteinuria that is rarely followed by azotemia (Cianciolo et al., 2018).

Juvenile nephropathies (like *Alport syndrome-like nephropathy, collagenofibric glomerulopathy, and renal maldevelopment*) have non-inflammatory, degenerative, or developmental backgrounds where the patients develop CKD before or around two years of age. It can be hereditary or acquired. Many breeds are predisposed (see Table 2) (Littman, 2011). The LM picture is generally not pathognomic with primary glomerular changes and secondary tubulointerstitial injuries. Different types of hereditary glomerulopathies have been described, and in some of them, genetic background has also been identified (Cianciolo et al., 2018). Because of the genetic mutations, abnormal molecules (usually proteins) are produced in the glomerular capillary wall. This defect in the integrity of the glomerular filtration barrier (GFB) either causes an immediate malfunction of the permselectivity, or the GFB will be more susceptible to injuries, or the deposition of circulating immune complexes will happen easier. The resulting clinical picture is a protein-losing nephropathy (PLN) with mild to severe proteinuria (Littman, 2011).

In *Alport syndrome-like nephropathy*, an abnormal production and assembly of type IV collagen (one of the essential components of the GBM) results in structurally abnormal GBM and glomerulosclerosis. The LM picture is not specific; glomeruli are hypercellular, and GBM is irregularly thickened. On EM, the irregular GBM thickening and splitting are characteristic of this disease (Cianciolo, 2018). Various mutations in the COL4A3, COL4A4, and COL4A5 genes cause an abnormal production of subtypes of alpha3-5 chains of collagen IV. More than 350 mutations have been identified in people. Some of them also affect the inner ear and the eyes. In dogs, *Alport syndrome-like nephropathy* and the mode of inheritance (autosomal recessive/dominant or X-linked) has been identified in bull terrier, Dalmatian,

English cocker spaniel, English springer spaniel, Samoyed and Navasota mix-breed dogs (see Table 2). The inheritance in Samoyed and Navasota mix-breed dogs is X-linked (Littman, 2011). In all dogs with Alport-like syndrome, proteinuria starts at a young age (3-6 months). In males with X-linked hereditary nephropathies and all dogs with autosomal recessive inheritances, the kidney disease rapidly progresses and terms in an end-stage CKD at 6-24 months of age. The progression of the glomerulopathy is a lot slower in carrier females with X-linked inheritance. Although they are also proteinuric, kidney failure usually only develops over five years of age (Lees, 2013).

Table 2. Breeds predisposed to glomerular pathogenic proteinuria. FSGS= focal segmental glomerulosclerosis, IMGN=immune complex-mediated glomerulonephritis, MCD=minimal change disease, MPGN=membranoproliferative glomerulonephritis (Littmann, 2011).

Breed	Disease
American foxhound	MPGN secondary to leishmaniasis
Basenji	Glomerulopathy with small intestinal immunoproliferative disease
Beagle	Primary glomerulopathy, amyloidosis
Bernese mountain dog	MPGN
Boxer	Reflux nephropathy with segmental hypoplasia
Brittany spaniel	Primary glomerulopathy
Bull terrier	Primary glomerulopathy
Bullmastiff	FSGS
Dalmatian	Hereditary nephropathy
Doberman pinscher	Primary glomerulopathy IMGN caused by sulfonamides
English cocker spaniel	Hereditary nephropathy
English foxhound	Amyloidosis
French mastiff (Bordeaux)	Juvenile glomerulopathy
German shepherd	IMGN (MCD) secondary to Ehrlichia canis infection
Golder retriever	IMGN caused by Lyme nephritis Juvenile renal disease
Gordon setter	Juvenile nephropathy
Greyhound	GN vasculopathy (skin, renal)
Labrador retriever	IMGN caused by Lyme nephritis
Mixed Navasota dog and kindred	Primary glomerulopathy
Newfoundland	Glomerulosclerosis
Norwegian elkhound	Periglomerular fibrosis plus tubulointerstitial disease
Pembroke Welsh corgi	Primary glomerulonephropathy
Rottweiler	Primary glomerulopathy
Samoyed and kindred	Primary glomerulopathy
Shar-Pei	Amyloidosis
Shetland sheepdog	IMGN caused by Lyme nephritis
Soft-coated wheaten terrier	FSGS vs IMGN Juvenile nephropathy

Collagenofibric glomerulopathy affects all glomeruli and it is characterized by massive accumulations of type III (and V) collagen fibrils in the GBM. Similarly to amyloidosis the LM picture shows global expansion of the mesangium and capillary loops by eosinophilic material (collagen), but it is not congophilic as amyloid. In the EM picture large cross-banded fibrils within the widened GBM and mesangium can be seen (see Figure 5) (Cianciolo, 2018).

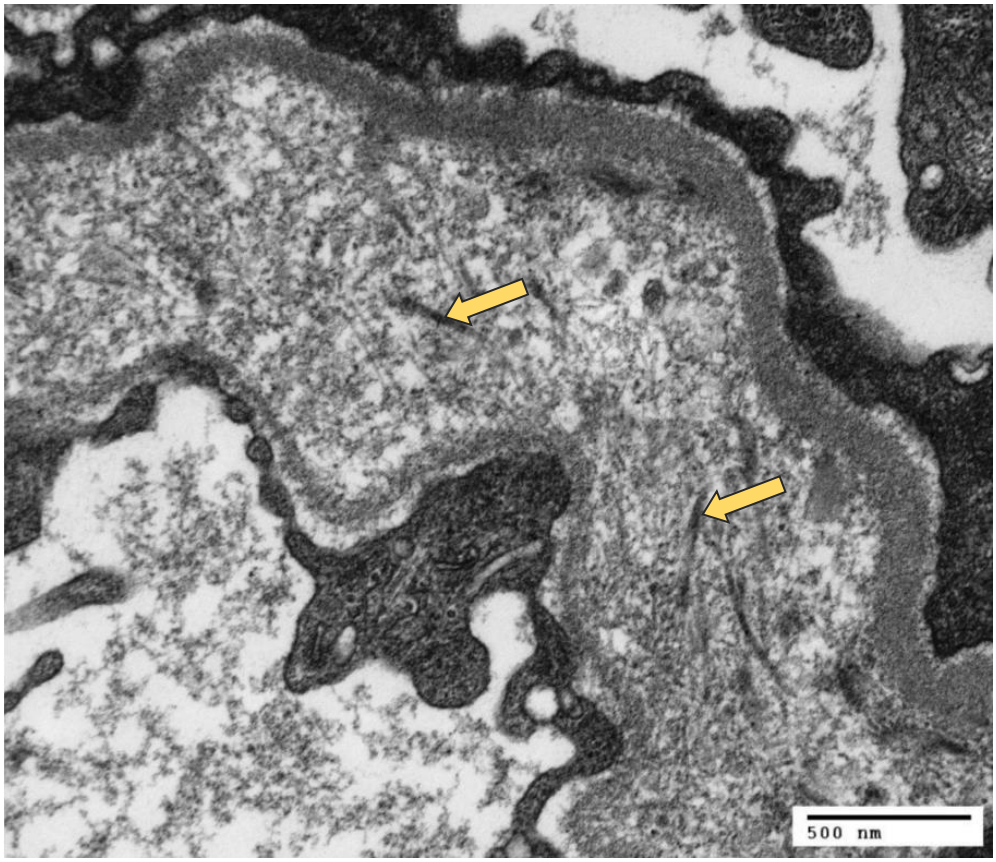

Figure 5. Transmission electron microscopic picture of a glomerulus with collagenofibric glomerulopathy. "The glomerular basement membrane is markedly widened and contains variably sized fibrils and granular material. There is podocyte foot process effacement (Cianciolo et al. 2018)."

Renal maldevelopment (renal dysplasia) means the disorganized development of renal parenchyma. It is not one specific disease but rather "diverse disorders that are characterized by mostly developmental anomalies and/or lesions". LM shows fetal or immature glomeruli (small and poorly capillarized) and tubules (small, dilated) that can be accompanied by interstitial fibrosis and inflammation (Cianciolo et al., 2018).

Four extensive studies describe the prevalence of the different types of glomerulopathies in canine (2) and feline (2) kidney biopsies. In all studies, the ratio of immune-mediated vs. non-immune-mediated diseases was around 50%. Cianciolo et al. examined 89 canine kidney samples. 26% of the dogs had membranoproliferative GN, 26% had membranous

GN, 13% had amyloidosis, and 29% had FSGS (Cianciolo et al., 2016). Aresu et al. also compared the morphological diagnoses with clinical and laboratory variables. 71/130 (54.6%) dogs had ICGN. 12/130 (9.2%) dogs had membranous GN, 27/130 (20.8%) had membranoproliferative GN, 16/130 (12.3%) had mixed GN, 16/130 (12.3%) had FSGS II. 7/130 (5.4%) dogs had amyloidosis, 30/130 (23.0%) had FSGS I, 6/130 (4.6%) had juvenile nephropathy, and 5/130 (3.8%) had minimal change disease. They found that UPC was higher in the ICGN group (9.28±10.05) than in the non-ICGN (4.73±2.82) and RL-NOS (2.98±3.28) groups, indicating that immune complexes cause a higher increase in glomerular permselectivity than other diseases. From a clinical point of view, it was interesting that no dogs with non-ICGN and RL-NOS had a UPC >12.5, while in the ICGN group, 21% of the dogs had UPC >12.5, with a maximum of 69.0. Parallel to this, serum albumin was markedly decreased in the ICGN group (20.10±6.92 g/L), while it was 24.01±7.38 g/L in the non-ICGN and 27.99±5.36 in the RL-NOS groups (Aresu et al., 2017).

Rossi et al. examined renal biopsy samples of 68 cats. Due to the limited number of samples and limited knowledge of feline glomerulopathies, the cat disease categories are still not as well described as in dogs. Cats were only separated into two groups: ICGN and non-ICGN (including all non-immune-mediated diseases). 37/68 cats (54.4%) had ICGN and 31/68 (45.6%) non-ICGN. In the ICGN group, 18/37 (48.6%) cats were diagnosed with membranous GN, 14/37 (37.8%) with membranoproliferative GN, and 5/37 (13.5%) mesangioproliferative GN. In the non-ICGN group, 11/31 (35.5%) cats had end-stage CKD, 9/31 (29%) had FSGS, 6/31 (19.4%) had global and multifocal mesangiosclerosis, 2 (6.5%) glomerular atrophy, 2 (6.5%) renal dysplasia and 1 (3.1%) amyloidosis. Cats in the ICGN group were younger: 8.9±3.5 years vs. 10.3 ± 3.4 years compared to the non-ICGN group. Cats with ICGN were more frequently infected with FIV or FeLV and had higher UPCs: 7.5±3.5 vs. 2.8 ± 0.9 in the non-ICGN group (Rossi et al., 2012).

The study of Rayhel et al. analysed the data of 58 proteinuric cats with clinicopathologic data and histopathology reports. Forty-two cats had protein-losing nephropathy (PLN). 31/42 (73.8%) had ICGN, and 11/42 (26.2%) had other glomerulopathies. Amyloidosis was present in 2/42 (4.8%) cats. UPC was higher in cats with other glomerulopathies (median 14.5) than in cats with ICGN (median 6.5). Cats with ICGN were older (median 3.5 years) than cats with other glomerulopathies (median 1.3 years). Males were overrepresented in the ICGN group (Rayhel, 2020). The disagreement between the two cat studies is probably due to the small sample sizes.

5.4 Detection of proteins in the urine

Several methods can be used for the detection of proteinuria. The simplest and least expensive is the **urinary dipstick test**. It is a semiquantitative, colorimetric test that can detect protein levels above 300 mg/L. It is widely used in veterinary practices. Its disadvantage is the poor sensitivity and specificity to albumin. In the study of Lyon et al., the specificity for albumin was 81.2% in dogs while only 11% in cats. The positive predictive value was 34% in dogs and 55.6% in cats. (Lyon et al., 2010) Another study found that the dipstick test could only detect proteinuria reliably if the UPC was>4 (Mamone et al., 2014). False positive reactions occur frequently due to high urinary concentration, pigmenturia, active urine sediment (pyuria, bacteriuria, hematuria), and late read tests. The reasons for false negative results could be dilute urine, the presence of Bence-Jones proteins, or small amounts of protein. False positive reactions are more frequent in alkaline urine, while false negative results are more common in acidic urine (Grauer, 2011; Harley & Langston, 2012).

The **sulfosalicylic acid (SAA) test** is a simple, semiquantitative test based on turbidimetric principles. It is performed by mixing equal parts of urine supernatant and 5% sulfosalicylic acid in a glass tube. Urinary proteins precipitate and make the solution turbid. The turbidity is subjectively graded from 0 to ++++. The advantage of this test is that it also detects globulins and Bence-Jones proteins. Its disadvantage is that its sensitivity and specificity are still insufficient for clinical practice. False positive reactions can occur due to radiographic contrast agents, penicillins, cephalosporins, certain volatile oils (e.g. thymol), or sulfonamides. There are fewer false negative results than at the dipstick, as it detects proteins over 50 mg/L (Grauer, 2011; Harley & Langston, 2012; Mamone et al., 2014).

If the urinary dipstick or sulfosalicylic acid test suggests proteinuria, its magnitude and persistence should be quantified. In people, proteinuria is measured in a 24-hour collected sample or urinary protein is normalized to urinary creatinine and expressed as **urinary protein-to-creatinine ratio (UPC)**. As creatinine is freely filtered through the glomeruli and is not reabsorbed, it reflects the urine concentration well. Normalizing to creatinine can prevent the distorting effect of different urine concentrations. The disadvantage of this method is that extreme changes in muscle mass can cause false low (e.g., significant muscle mass) or false high (e.g., muscle wasting, neuromuscular disease) UPC results. When the two methods were compared, it was shown that measuring UPC from spot urine is accurate for clinical use. However, for the follow-up of severe proteinuric patients, 24-hour urine collection is recommended (Bökenkamp, 2020). As it is challenging to collect 24-hour urine samples in veterinary patients, the gold standard test for quantifying proteinuria is to assess the UPC of a spot urine sample (Grauer, 2011). It was proven that a UPC from a single urine sample correlates well with the protein content of a 24-hour sample (Adams, 1992).

Based on the International Renal Interest Society (IRIS) classification, UPC <0.2 is considered physiologic in dogs and cats, UPC between 0.2-0.4 in cats and 0.2-0.5 in dogs is called borderline proteinuria, and UPC ≥0.4 in cats and ≥0.5 in dogs is considered proteinuria (Lees et al., 2005).

Proteinuria is considered persistent if detected in 3 or more different urine samples at least two weeks apart. Generally, glomerular proteinuria causes higher UPC, while tubulopathies are followed by less severe protein loss (UPC typically stays under 1-2). A UPC that is persistently higher than 2 is highly suspicious for glomerulopathy (Lees et al., 2005).

When assessing the UPC, it is essential to avoid preanalytic errors. Beatrice et al. compared the UPC results from 230 paired urine samples (from 115 dogs) taken via cystocentesis and free-catch methods. They found that 92.6% of the dogs were categorised in the same IRIS proteinuria substage with the two methods, and there was no significant difference between the results (Beatrice et al., 2010). Vilhena et al. examined the same on cats. They found that all 43 cats were categorized into the same IRIS proteinuria substage when UPC was compared in cystocentesis and free-catch samples (Vilhena and Santos, 2015). In contrast, a similar study from Mortier et al. found that 28% of the 92 cats were categorised into different IRIS proteinuria substages (Mortier et al., 2023). Duffy et al. compared the UPC from free-catch urine samples collected in home and hospital settings. They found that UPC was higher in 50% of the 24 paired samples taken in the hospital setting (Duffy et al., 2015). Based on these studies, it is advised to always take the urine samples in the same place and use the same method. Nabity et al. examined day-to-day variations of the UPC. They collected urine samples on three consecutive days. They found that if UPC is <4, one sample can provide a reliable result, but if UPC is >4, day-to-day variation is high, and taking the average of more UPCs could give us more reliable information about proteinuria. They suggested taking the average of the UPCs of 2-3 samples if UPC is between 4 and 8, and 4-5 measurements if UPC >8 (Nabity et al., 2007). If measuring serial UPCs from different urine samples is not feasible financially, using pooled samples (by mixing equal quantities from serial urine specimens) is a reliable and cost-effective alternative (LeVine et al., 2010). Rossi et al. examined the changes in the urine protein content when stored at different temperatures. They stated that UPC was unchanged at room temperature for 4 hours, but protein content increased significantly after 12 hours. At 4^{o}C, there was a mild elevation after 12 hours and significant elevation after one week. UPCs of samples stored at -20 ^{o}C increased after one week and two months, although the magnitude of this increase was clinically unimportant (Rossi et al., 2012).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method that separates proteins by their molecular weight and charge. It can help determine the origin of renal proteinuria (glomerular or tubular). Larger-sized molecules (e.g., albumin) indicate glomerular damage, while smaller-sized proteins indicate tubular problems (Littman, 2011).

The detection of albuminuria and UAC (albumin-to-creatinine ratio) is discussed in Chapter 6.1.

5.5 Clinical presentation of proteinuric patients

Proteinuria is frequently seen in middle-aged to older dogs (average 8.3 years), although it can be diagnosed at any age. In cats, proteinuria is less common than in dogs (Vaden, 2011). Some authors found a slight male predominance. Patients with proteinuria can be asymptomatic for a long time. Littman suggests a four-stage categorisation for the progression of protein-losing nephropathy (PLN) (this is different from the IRIS four-stage categorisation for CKD). **In Stage 1,** persistent glomerular proteinuria is present without other kidney-related symptoms. Symptoms related to other underlying diseases (see Table 1) could be present (Littman, 2011).

In Stage 2, the glomerulopathy progresses, UPC increases, hypoalbuminemia develops, and the typical complications of proteinuria appear: hyperlipidemia, hypercoagulability, hypertension, hypoproteinemia, and extravascular fluid accumulation. These consequences can be present separately or in combination. Although it is a severe kidney disease, there is still no azotemia in this stage (this equals Stage 1 CKD in the IRIS staging system) (Littman, 2011).

Hyperlipidemia results from decreased cholesterol breakdown due to urinary loss of the lecithin-cholesterol acyltransferase enzyme. At the same time, the liver increases the synthesis of some lipoproteins, leading to decreased high-density lipoprotein and increased low-density lipoprotein and triglycerides (Littman, 2011).

Hypertension has many reasons in patients with proteinuria. The renin-angiotensin-aldosterone system is activated, and the kidneys produce fewer vasodilators (prostaglandins and kinins). Sodium retention, glomerular capillary scarring, and increased arterial sensitivity to vasoconstrictors also contribute to hypertension. Hypertension can damage four organ systems, the so-called target organs: the eyes, the central nervous system, the cardiovascular system, and the kidneys. Acute blindness because of retinal bleeding and detachment and papilledema is a typical reason for veterinary visits in hypertensive patients. Hemorrhages can affect the central nervous system as well. Cardiovascular consequences include left ventricular hypertrophy, epistaxis, arteriosclerosis, and sometimes aortic dissection (Oricco et al., 2019).

Renal consequences are progressive nephron loss, worsening proteinuria, glomerulosclerosis, and pressure diuresis. Hypertension is categorized by the risk of target organ damage (see Table 3) (Acierno et al., 2018; Littman, 2011).

Table 3. Categorisation of hypertension by the risk of target organ damage (Acierno et al., 2018).

Systolic Blood Pressure	Blood Pressure Substage	Risk of Future Target Organ Damage
<140	Normotensive	Minimal
140-159	Prehypertensive	Low
160-179	Hypertensive	Moderate
≥180	Severely hypertensive	High

Hypercoagulability increases the risk of both arterial and venous thromboembolic events in proteinuric patients. A study by Cook et al. found that 22% of dogs with protein-losing nephropathy had thromboembolic events (Cook et al., 1996). Lennon et al. compared the coagulation tendency in 28 dogs with PLN with eight control dogs. Thromboelastography showed hypercoagulability in the PLN group. Interestingly, there was no correlation between hypercoagulability and serum albumin levels or the UPC (Lennon et al., 2013). Hypercoagulability has multifactorial causes: loss of antithrombin III through urine, mild thrombocytosis, platelet hypersensitivity (increased aggregation and adhesion), and changed fibrinolysis (Rothrock, 2016; Littman, 2011).

Many possible mechanisms lead to fluid extravasation, and most likely, more of them are present simultaneously. It can result from decreased plasma oncotic pressure due to protein loss, increased intravascular hydrostatic pressure because of hypernatremia, or increased vascular (endothelial cell) permeability (Klosterman & Pressler, 2011).

Nephrotic syndrome is the clinical presentation of a severe glomerulopathy where proteinuria, hypercholesterolemia, hypoalbuminemia, and interstitial or third-space fluid accumulation (e.g., ascites, thoracic free fluid) are present. Nephrotic syndrome is present in around 15% of dogs with glomerulopathies, while incomplete nephrotic syndrome (without fluid accumulations) is present in 49% of dogs (Vaden, 2011). Klosterman et al. compared the clinicopathologic data of 78 nephrotic glomerulonephropathy dogs with 156 dogs with non-nephrotic glomerulopathy. They did not find a correlation between the presence of nephrotic syndrome and histopathologic diagnosis. In the nephrotic group, serum albumin concentration was lower (median 16 vs. 27 g/L), UPC was higher (15.2 vs. 6.2), cholesterol was higher (353.2 vs. 290 mg/dL), chloride was higher (117 vs. 113 mmol/L), phosphate was

higher (6.8 vs 5.1 mg/dL), and calcium was lower (9.1 vs 10.1 mg/dL) than in the non-nephrotic group. There was no difference in the degree of azotemia, urine-specific gravity, and coagulation status (Klosterman et al., 2011).

Besides the complications mentioned above, proteinuria causes further glomerular and tubulointerstitial damage and accelerates the nephron loss. In people, proteins that accumulate within the glomerulus stimulate mesangial cell proliferation, while proteins in the ultrafiltrate are toxic to the tubular cells and cause interstitial inflammation, fibrosis, and tubular necrosis (Grauer, 2011).

In **Stage 3**, azotemia begins (this stage equals Stage 2-3 CKD in the IRIS staging system). UPC may decrease in this stage, but it is only because fever nephrons are working. In **Stage 4**, the disease progresses into end-stage renal disease with severe azotemia (IRIS Stage 4 CKD) and the typical symptoms of CKD (PU/PD, vomiting, anorexia, weight loss). Proteinuria may decrease further, hypoalbuminemia may improve, or dehydration can mask it in this stage (Littman, 2011).

5.6 Diagnostic workup of proteinuric patients

The diagnostic workup should start with signalment and history. The inspection of breed predispositions (Table 2) and family history can help in the diagnosis of familial nephropathies.

The thorough history should include information about symptoms related to kidney disease (polyuria, polydipsia, gastrointestinal signs, inappetence, weight loss), lower urinary tract signs (pollakiuria, hematuria, stranguria), other symptoms (lameness, dyspnea, loss of sight, neurologic events), prior illnesses (allergies, inflammatory bowel disease, protein-losing enteropathy, tick-born illnesses), travel, medication (e.g. masinitib), vaccination, tick exposure and prevention.

The physical examination should focus on body condition, hydration status, mucose membranes (anemia, petechiae), peripheral edema, ascites, respiratory and cardiologic alterations, lymphadenomegaly, joint swelling, and abdominal palpation (organomegaly). An ophthalmologic (fundic) exam is necessary when problems with vision are noted, searching for signs of hypertension (e.g., torturous retinal vessels, retinal damage).

Possible additional diagnostic tests include blood pressure measurement, laboratory tests (blood, urine), imaging (ultrasound, X-ray), and renal cortical biopsies. A basic laboratory evaluation containing a complete blood count and general biochemical profile is always needed. Testing for infectious diseases depends on the specialties of the geographic area (borreliosis, dirofilariasis, ehrlichiosis, anaplasmosis, bartonellosis, babesiosis, leptospirosis, brucellosis, leishmaniasis, trypanosomiasis, Rocky Mountain spotted fever).

Coagulation profile, thromboelastography, and blood cultures can be carried out if necessary. Coombs, antinuclear antibody titer, rheumatoid factor, perinuclear antineutrophil cytoplasmic antibody, circulating immune complex, and complement-3 tests could be done if an immune-mediated process is suspected. Urinalysis should include dipstick, sediment, and UPC evaluation. Urinary albumin-to-creatinine ratio, urine culture, and SDS-PAGE electrophoresis may also be added for a more complete assessment of the urine sample. Cytologic evaluation of effusions, enlarged lymph nodes, bone marrow, or joints could help to find an accurate diagnosis (Littman, 2011).

5.7 Therapy of Proteinuria

Transient proteinuria necessitates no treatment; it resolves itself. If proteinuria is persistent, standard therapy should be initiated. It is based on a clinical renal diet, medication blocking the renin-angiotensin-aldosterone axis, antihypertensive and anticoagulant treatment, and sometimes fluid or diuretic therapy. If an underlying disease is diagnosed, it should be treated according to its nature (antibiotics for bacterial infections, antiparasitics against *Dirofilaria* and *Babesia* infections, chemotherapy for neoplastic diseases, etc.) Specific therapy of glomerulopathies includes immunosuppressive therapy for immune-mediated glomerulonephritis and colchicine for (preventing) amyloidosis (Littman, 2011).

5.7.1 Standard therapy of proteinuria

Standard therapy is recommended for all dogs and cats with renal proteinuria regardless of the underlying etiology and disease severity. It should be initiated when UPC exceeds 0.5. The therapy's general aim is to reduce the UPC to <0.5, but as it is often not possible, a 50% reduction in the UPC is a realistic goal (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013).

5.7.1.1 Dietary therapy

Nutrition is one of the critical factors in the management of proteinuric kidney diseases. Diets formulated for renal diseases have been used in veterinary medicine for over 70 years. Generally, renal diets contain less proteins, phosphorus and sodium, and higher amounts of vitamin B-complexes, buffering agents, antioxidants, and omega-3 fatty acids. (Roudebush et al., 2010). A well-set protein content and polyunsaturated fatty acids (PUFA) can decrease proteinuria. Feeding a clinical renal diet can significantly prolong the life of dogs and cats with CKD. Renal diets contain the highest quality but moderately reduced amount of proteins.

High amounts of dietary proteins aggravate urinary protein losses. The amount of circulating protein can decrease with the reduction of protein intake, so the protein load on the glomeruli will be lower, and the risk of overwhelming the glomerular filtration barrier and the tubular reabsorptive capacity decreases, as does proteinuria. Care should be taken not to restrict protein intake severely because it can lead to weight loss and hypoalbuminemia. (Burkholder, 2004; Littman, 2011; IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013; Sparkes et al., 2016; Zatelli et al., 2016).

It has long been known that omega-3 fatty acids (docosahexaenoic acid and eicosapentaenoic acid) have many positive effects on the kidneys in people. Docosahexaenoic acid and eicosapentaenoic acid compete with arachidonic acid and form eicosanoids that have anti-inflammatory, antioxidant, and cholesterol-decreasing effects and positive effects on circulation. For example, instead of thromboxane A2, thromboxane A3 will be produced, a less potent vasoconstrictor, while prostacyclin A3 has the same vasodilatory effect as prostacyclin A2. The net effect of these molecules is vasodilation of renal vessels, thus reducing intraglomerular pressure and proteinuria (Mori, 2004; Roudebush et al., 2010). Omega-3 PUFA supplementation was also shown to have renoprotective effects in laboratory dogs with induced CKD. It decreased mortality, helped maintain renal function, and decreased proteinuria and serum cholesterol levels. Renal lesions like glomerulosclerosis, mesangial matrix expansion, tubulointerstitial fibrosis, and inflammation also diminished (Brown, 1998). Omega 6:3 PUFA ratio in commercial dog and cat food is around 50:1, while the recommended ratio for dogs and cats with CKD is 5:1 (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013).

Based on laboratory studies, dietary sodium restriction is also suggested for dogs with glomerulopathy. Generally, dogs are not salt-sensitive like people. However, it is possible that dogs with CKD, especially dogs with nephrotic syndrome, are salt-sensitive and may benefit from the antihypertensive and renal hemodynamic consequences of salt restriction (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013).

5.7.1.2 Antiproteinuric therapy

The medical treatment of proteinuria is based on the inhibition of the renin-angiotensin-aldosterone system (RAAS). The RAAS is activated by hypotension, dehydration, fall in cardiac output, sodium depletion, and the sympathetic nervous system. The juxtaglomerular epithelial cells answer to these stimuli with the release of renin. The activation of the RAAS is controlled at this site, as angiotensinogen is continuously present in the circulation (Ames, 2018). The most important vasopeptid of the system is angiotensin II (Ang II) which has many harmful effects in the progression of chronic kidney diseases and proteinuria. Renin

inhibitors and angiotensin-converting enzyme inhibitors (ACEis) block the formation of Ang I or II, while angiotensin receptor blockers (ARBs) block the type 1 angiotensin receptor, thus Ang II can not bind to its receptor (see Figure 6). Renin inhibitors are only used in human medicine while ACEis and ARBs are widely used in veterinary medicine (IRIS Canine GN Study Group Standard Therapy subgroup et al, 2013).

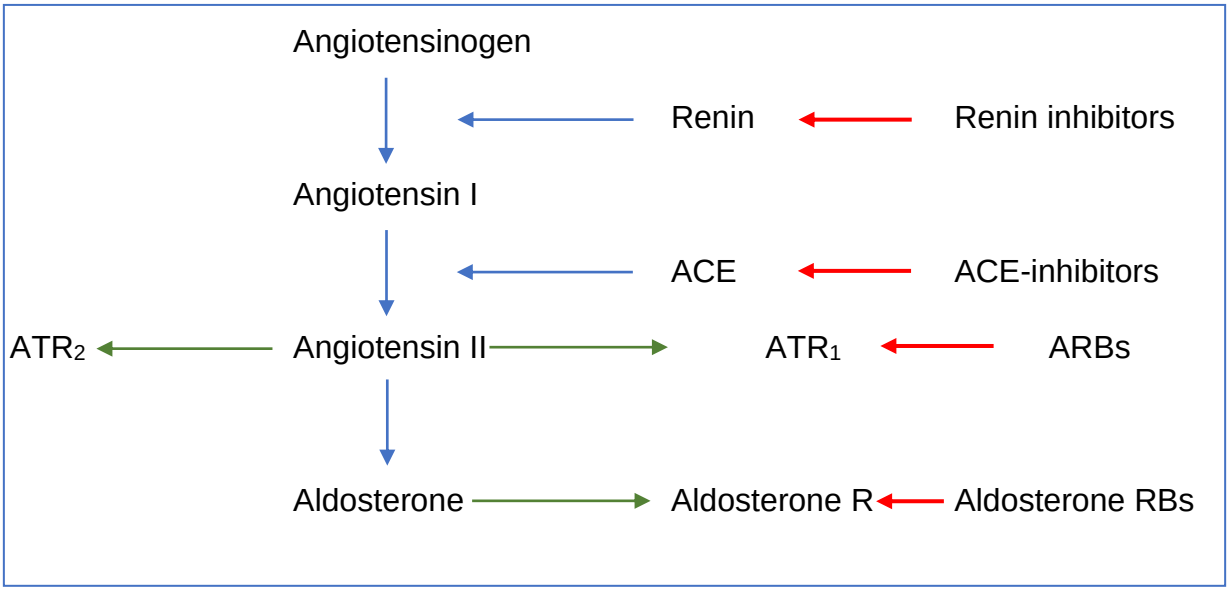

Figure 6. Renin-angiotensin-aldosterone axis and its possible inhibition. ACE=angiotensin-converting enzyme, ARB=angiotensin receptor blocker, ATR=angiotensin receptor, RB=receptor blocker (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013).

The RAAS is responsible for sodium and water homeostasis and long-term maintenance of normal blood pressure. Ang II can increase blood pressure as a physiologic response to systemic hypotension. It causes systemic vasoconstriction and stimulates aldosterone release from the adrenal glands, thus causing sodium and water retention. It is known that the activity of the RAAS increases in people and animals with kidney diseases. Ang II acts not only on the systemic blood pressure but also on the local blood pressure regulation within the glomeruli. Ang II causes vasoconstriction on the efferent arteriole, thus increasing intraglomerular pressure and glomerular filtration rate (GFR). In the short term, it is a physiologic response to the decreased renal blood flow. However, in the long term, it leads to worsening proteinuria and progressive nephron loss because of hyperfiltration. In addition, Ang II has proinflammatory and profibrotic effects (Wolf & Ritz, 2005).

ACEis (e.g., enalapril, benazepril, ramipril) are widely used in veterinary medicine. Their effectiveness in reducing proteinuria is proven in dogs and cats (Brown, 2001; Brown, 2003; Tenhundfeld, 2009). There is no evidence that one ACEi would be superior to another. A

study found enalapril more effective than benazepril. However, this result is questionable as it was given in a higher dose, and the study was supported by the manufacturer of enalapril (Zatelli et al., 2016). The advantage of benazepril is that it is excreted mainly by the liver, while enalapril is eliminated through the kidneys. A dose reduction of enalapril is needed in case of severe azotemia.

ARBs are another commonly used type of RAAS inhibitor. Telmisartan seems to be the most effective ARB in veterinary patients, while the use of losartan is declining. A study found that although dogs do not produce the most crucial active metabolite of losartan, it can still be effective, but it is ineffective in cats (Vaden, 2016). Recent studies showed that telmisartan is more effective in reducing proteinuria than ACEis (Fowler et al., 2021; Lecavalier et al., 2021; Lourenço et al., 2020). In 2023, the IRIS changed its recommendation, and telmisartan became the first-line drug for treating proteinuria in dogs (http://iris-kidney.com/guidelines/guidelines_updates_2023.html).

Previously, ACEis and ARBs were frequently used in combination therapy in human medicine. Recently, it was shown that despite the more pronounced albumin-lowering effect, combination therapy provides no benefit for the kidneys and increases the risk of hyperkalaemia and acute kidney injury (Sharma and Smyth, 2021).

Aldosterone breakthrough means that after long-term use of ACEis or ARBs, aldosterone levels start to increase because of the activation of some non-ACE-dependent Ang II-producing pathways. In people, aldosterone receptor blockers (e.g., spironolactone) are used when this happens. In those cases, the inhibition of aldosterone receptors can help in the further reduction of proteinuria. There is limited data on the antiproteinuric effects of spironolactone in dogs and cats. As it is a well-tolerated drug, its use should be considered in cases that are not responding well to ACEis or ARBs (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013; Wolf & Ritz, 2005).

All drugs blocking the RAAS can cause severe side effects: hyperkalemia, worsening azotemia, or even severe acute kidney injury (especially if given to dehydrated animals) and hypotension. Close monitoring is needed to observe these side effects promptly. Serum creatinine and potassium levels, and blood pressure should be measured 1-2 weeks and 4-6 weeks after every dose adjustment or drug change (see Figure 7). Hyperkalemia should be closely monitored when it is over 6 mmol/l, and changes in medication have to be done if potassium is >6.5 mmol/l to prevent cardiotoxicity. A small extent of creatinine elevation from baseline is allowed: <30% in IRIS Stages 1 and 2 CKD, <10% in IRIS Stage 3, and no elevation is allowed in IRIS Stage 4 (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013).

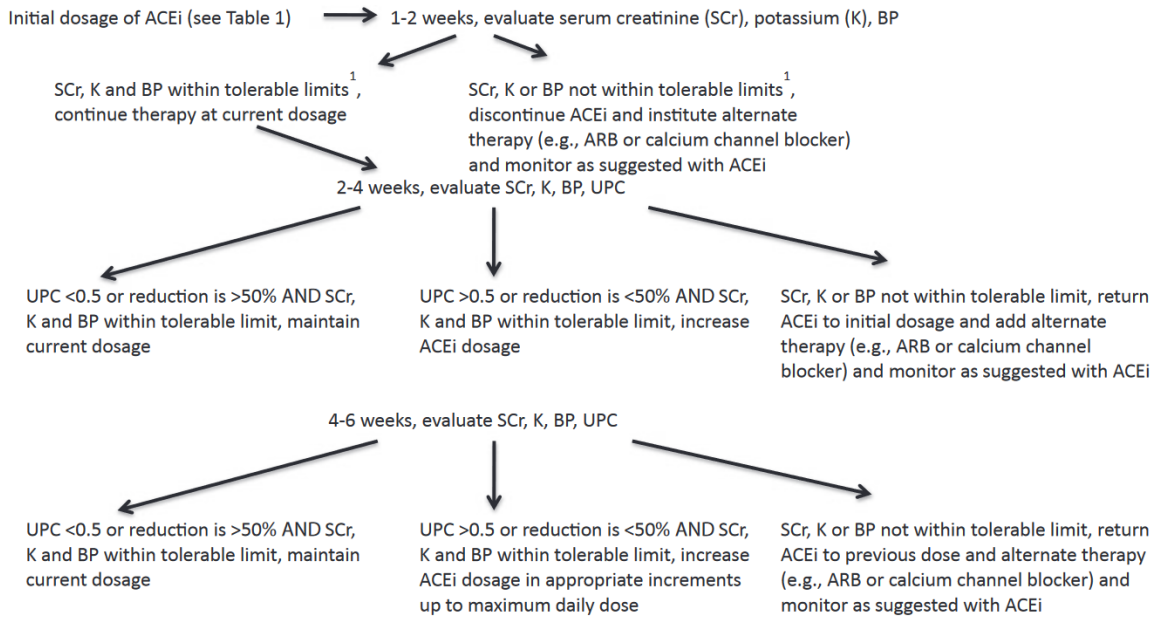

Figure 7. Making adjustments to RAAS inhibition therapy in dogs. SCr=serum creatinine, BP=blood pressure (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013.)

5.7.1.3 Antihypertensive therapy

Drugs blocking the RAAS can lower blood pressure as well. ACEis are mild, while ARB telmisartan has a moderate antihypertensive effect (Coleman et al., 2019; Fowler et al., 2021). If hypertension (systolic >160 Hgmm) is still present in a proteinuric patient despite the blockage of the RAAS, the calcium channel blocker amlodipine should be added to the therapy. In case of severe systemic hypertension or diagnosed target organ damage, amlodipine therapy should be initiated immediately. The goal of antihypertensive therapy is to reduce the risk of target organ damage and to lower the systolic blood pressure <150 Hgmm. After every drug adjustment, blood pressure should be reevaluated within 3-14 days in IRIS Stages 1-2 of CKD and within 3-5 days in IRIS Stages 3-4 or in unstable patients. After setting the treatment, blood pressure should be controlled every 1-4 months (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013).

5.7.1.4 Anticoagulant therapy

Anticoagulant therapy is recommended for dog and cat patients with severe PLN. The IRIS recommends it if serum albumin level is <20 g/L, while other authors recommend it as soon as hypoalbuminemia develops (Harley and Langston, 2012.; IRIS Canine GN Study

Group Standard Therapy Subgroup et al., 2013). Previously, a low dose of acetylsalicylic acid (Aspirin) was the first-line medication for thrombosis prevention in PLN. Acetylsalicylic acid decreases the production of thromboxane A2 and thus decreases thrombocyte aggregation. Recently, clopidogrel became the first choice for thromboprophylaxis in dogs and cats (http://iris-kidney.com/guidelines/guidelines_updates_2023.html). Clopidogrel binds to the ADP receptor of platelets and inhibits platelet aggregation (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013).

5.7.1.5 Fluid and diuretic therapy

Dogs and cats with CKD often get dehydrated and require fluid therapy. Imbalances in fluid homeostasis are also common in patients with glomerulopathies, but they can be very variable. Hypovolemia, dehydration, hypervolemia, or intercompartmental maldistribution can happen. Intercompartmental maldistribution means that a patient can get hypovolemic or dehydrated despite excessive fluid accumulation, e.g., in the abdominal or thoracic cavity. Fluid therapy should be, therefore, personalized and set based on anamnestic data (prior and actual body weight), physical examination (skin turgor, mucose membranes, capillary refilling time, pulse quality, heart rate), and blood pressure measurement. For fluid therapy, crystalloids should be given cautiously and slowly, with frequent reevaluations. The effectiveness of colloid infusion therapy in nephrotic syndrome is not proven. It is not advised to administer colloids routinely just based on the presence of hypoalbuminemia, neither in people nor in dogs or cats, because of its possible tubulotoxic effect. It can be given cautiously if the patient is still hypovolemic after crystalloid infusion treatment.

In case the patient is hypervolemic, cautious diuretic therapy is suggested. The loop diuretic furosemide is the first choice in pulmonary edema, and the aldosterone blocker spironolactone is recommended in case of thoracic or abdominal free fluid (IRIS Canine GN Study Group Standard Therapy Subgroup et al., 2013).

5.7.2 Immunosuppressive treatment of proteinuria

Immunosuppressive therapy is recommended in case of severe, persistent, or progressive glomerulopathy, where histopathology confirmed an immune-mediated pathogenesis (IRIS Canine GN Study Group Established Pathology Subgroup et al., 2013). As there is an around 50% chance of immune-mediated origin both in cats and dogs (Aresu et al., 2017; Rayhel et al., 2020), immunosuppressive treatment can be initiated as a therapy trial without established histopathology where:

- severe and persistent proteinuria is present (UPC is more than 2-3) or
- serum creatinine is > 260 µmol/l or azotemia is progressive or
- severe hypoalbuminemia (<20 g/L) is present.

There are several contraindications of immunosuppressive therapy:

- diabetes mellitus
- Cushing's disease
- fungal or bacterial infections
- dogs from breeds that are predisposed to familial nephropathies (see Table 2) (except soft-coated wheaten terriers and Bernese mountain dogs, because these breeds may benefit from immunosuppression)
- dogs from breeds where amyloidosis is of familial origin (see Table 2) (IRIS Canine GN Study Subgroup on Immunosuppressive Therapy Absent a Pathologic Diagnosis et al., 2013).

The choice of immunosuppressive medication should be made based on the severity of the glomerulopathy and its progression rate. The recommended dosages and adverse effects of immunosuppressive drugs are listed in Table 4. In peracute cases or where the disease is rapidly deteriorating (UPC is high, azotemia is worsening, presence of hypoalbuminemia or effusions), rapidly acting drugs are needed. Mycophenolate mofetil alone or combined with prednisolone is the first choice in these cases. Mycophenolate inhibits T-cell and B-cell proliferation by inhibiting the inosine monophosphate dehydrogenase enzyme needed for synthesizing guanosine nucleotides. Cyclophosphamide alone or with prednisolone is an alternative protocol. Glucocorticoids should not be used alone, and if administered in combination, the dose should be tapered down as soon as possible because of its various side effects. Besides the general side effects (polyuria, polydipsia, polyphagia, panting, muscle wasting, changes in fur quality, fat deposition), glucocorticoids have many side effects that are already present in glomerulopathies and thus can amplify these effects (thromboembolic risk, proteinuria, hypertension, sodium and water retention, urinary tract infection). Dogs with stable or slowly progressive disease can be treated with mycophenolate, chlorambucil alone, or combined with azathioprine, cyclophosphamide with glucocorticoids, or cyclosporin (IRIS Canine GN Study Group Established Pathology Subgroup et al., 2013).

Table 4. Recommended dosages and adverse effects of immunosuppressive drugs suggested for treating immune complex-mediated glomerulonephritis. (From IRIS Canine GN Study Group Established Pathology Subgroup et al., 2013)

Generic Name	Main Mechanism of Action	Recommended Dose	Main Adverse Effects
Azathioprine	Antagonizes purine metabolism	2 mg/kg PO q24h for 1–2 weeks, then 1–2 mg/kg q48h	GI upset, myelosuppression, acute pancreatitis, GI disorders, hepatotoxicity, infection, malignancy
Chlorambucil	Alkylating agent	0.2 mg/kg PO q24–48h	GI upset, myelosuppression
Cyclophosphamide	Alkylating agent	Pulse therapy 200–250 mg/m^2 every 3 weeks Continuous therapy—50 mg/m^2 PO 4 days/week	Myelosuppression, GI upset, hemorrhagic cystitis, infection
Cyclosporine	Calcineurin inhibitor	5–20 mg/kg PO q12h (consider tapering dose upward from low to higher doses to avoid gastrointestinal complications)	GI upset, gingival hyperplasia,
Mycophenolate	Antagonizes guanosine metabolism	10 mg/kg PO q12h	GI upset
Prednisolone (or other glucocorticoids at an appropriate dosage and route of administration)	Inhibition of PLA2, reduction in cytokines release, inhibition of neutrophils migration, down regulation of the Fc receptor	1 mg/kg PO q12h initially. Taper down as soon as possible	PU/PD, polyphagia, muscle wasting, panting, haircoat changes, weight gain, induction of liver enzymes, GI ulceration, lipidemias, infection, adrenal suppression, thromboembolism

Once immunosuppressive therapy is started, careful monitoring should be made, emphasizing the UPC, serum albumin, and creatinine concentrations. The first control examination is generally 1-2 weeks after therapy starts, then every two weeks for 2-3 occasions, then every four weeks for three months, then every three months. A complete response to therapy is when UPC decreases under 0.5, serum albumin increases to >25 g/L, and serum creatinine decreases to <124 µmol/l. In partial response, UPC decreases by >50% compared to the UPC after standard therapy; albumin increases to 20-25 g/L, and serum creatinine decreases by >25%. In patients where UPC is very high, a 35% reduction is also acceptable. Therapeutic failure means UPC is not decreasing more than 50%, serum albumin does not increase to >20 g/L, and serum creatinine concentration decreases <25%. Secondary therapeutic goals are improving blood pressure, resolving fluid accumulations, and stabilizing body condition.

Immunosuppressive treatment should be discontinued in case of severe side effects, e.g., gastrointestinal signs, pancreatitis, systemic infections, hepatic failure, and severe leukopenia. If side effects are absent or tolerable, the effectiveness of the therapy should be evaluated after 8-12 weeks. If there is no response, therapy should be discontinued. If there is a complete or partial response, therapy should be given for at least 12-16 weeks. The dose should be gradually tapered to the minimum effective dose (IRIS Canine GN Study Group Established Pathology Subgroup et al., 2013).

Dogs from areas where infectious diseases leading to proteinuria are endemic should be cautiously monitored for infection during immunosuppressive therapy (IRIS Canine GN Study Subgroup on Immunosuppressive Therapy Absent a Pathologic Diagnosis et al., 2013).

5.7.3 Treatment of amyloidosis

There is no specific treatment for amyloidosis because amyloid fibrils are insoluble and resistant to proteolysis. Colchicine blocks the release of SAA from the hepatocytes and thus can prevent amyloid formation. Its administration is recommended in Shar-Pei dogs with febrile episodes and swollen hocks, as Shar-Pei fever often precedes renal amyloidosis.

Some authors recommend using dimethylsulfoxide (DMSO), while other studies found it ineffective in renal amyloidosis. It may decrease SAA concentrations and inflammation, but evidence is lacking to support its regular use (Vaden, 2017).

5.8 Prognosis of proteinuria

As many different etiologies can lead to proteinuria, the prognosis can vary. Some glomerulopathies progress rapidly into end-stage kidney disease, while others are stable for years, and patients can live normal lives with the presence of proteinuria. Although glomerulopathies are often progressive diseases, spontaneous remission and response to immunosuppressive therapy followed by clinical cure can also occur (Vaden, 2011).

It has been proven in dogs and cats that proteinuria leads to quicker progression of CKD. Jacob et al. found that a lower UPC (<1) led to longer survival time (599 days) in dogs, and as UPC increased, median survival time shortened: 334 days for UPC 1-1.67; 289 days for UPC 2-2.77, and 255 days for UPC 3-15.8 (Jacob et al., 2005). Wehner et al. found similar survival times: dogs with physiologic UPC lived for 1009 days, while dogs with overt proteinuria survived only for 390 days (Wehner et al., 2008). Syme et al. examined cats with CKD and found that cats with higher UPC (>0.4) survived less (281 days) than cats with lower UPC (<0.4) (766 days) (Syme et al., 2006). Jepson et al. studied cats with hypertension and found that non-proteinuric hypertensive cats survived longer (490 days) than those with proteinuria (162 days) (Jepson et al., 2007).

Cook et al. examined the survival of 53 dogs with GN or amyloidosis. The median survival time was only 28 days, although some individuals lived for over three years (Cook, 1996).

Klosterman et al.'s study found that dogs with nephrotic syndrome and azotemia had the worst prognosis (median survival time was 12.5 days). In comparison, dogs with

glomerulopathy without nephrotic syndrome and azotemia had a median survival time of 605 days (Klosterman et al., 2011).

The prognosis of amyloidosis is poor. In a study, the median survival time was 28 days, and only 8.5% of the dogs survived for more than one year (Vaden, 2017). In another study, the mean survival time of dogs with amyloidosis was five days, and high serum creatinine was a negative prognostic factor (Segev et al., 2012).

A recent study examined the survival of proteinuric cats based on exact histopathologic diagnosis. Cats' survival ranged from 3 to 1848 days (median 94 days). The survival times did not differ between the ICGN group and non-immune-mediated glomerulopathies. Cats with fluid accumulations lived shorter (94 days) than cats without effusions (700 days). In the ICGN group, cats that received immunosuppression lived longer (244 days) than those that did not (17 days) (Rayhel et al., 2020).

6. Establishment of a reference interval for urinary albumin-to-creatinine ratio in dogs

6.1 Overview of albuminuria

Albumin is the most important serum protein. Because of its size (69 kDa) and negative charge, only small amounts (2-3 mg/dL) can pass through the intact glomerular wall into the ultrafiltrate. Most of it will be reabsorbed in the proximal tubules and degraded by lysosomes, and only <1 mg/dL is found in normal urine. Albuminuria means an abnormal loss of albumin in the urine (Grauer, 2011).

6.1.1 Importance of albuminuria in human medicine

Recently, albuminuria has gained huge importance in human medicine in detecting, staging, and managing acute and chronic kidney diseases, along with GFR. GFR measures kidney function, while albuminuria is a marker of kidney damage. In recent international recommendations, the importance of albuminuria overcame the importance of proteinuria. Detection of albumin in the urine is preferred because it is the dominant urinary protein lost in most kidney diseases, and the measurement of urinary total protein cannot be standardized because of its variable composition (KDIGO, 2013; Levey et al., 2015). When significant non-albumin proteinuria is suspected, specific protein assays are recommended (e.g., a1-microglobulin, monoclonal heavy or light chains) (KDIGO, 2013).

Albuminuria is a sensitive but non-specific marker of renal damage, as its level can also increase in many other diseases or conditions. Albuminuria is also a marker of generalized vascular dysfunction in people. Patients with albuminuria are at greater risk of cardiovascular events (e.g., myocardial infarction, stroke) than those without. The cardiovascular risk correlates with the level of albuminuria. Elevation of urinary albumin concentration is also associated with cardiovascular and all-cause mortality (Jarraya et al., 2013). Albuminuria is a good predictor of morbidity and mortality in critically ill patients, too (Gosling, 2003; Bakris & Molitch, 2014). Hypertensive patients show albuminuria, as well, and it is associated with the severity and duration of hypertension (Jarraya et al., 2013). The presence and magnitude of albumin in the urine can predict renal disease progression in diabetic patients, especially if combined with lower GFR (Bakris & Molitch, 2014). Although albuminuria should be considered a risk marker for renal disease progression and cardiovascular events in diabetic

patients, its presence alone does not mean kidney disease without a decline in GFR (Bakris & Molitch, 2014). Screening of albumin excretion is recommended in all patients who are at risk for kidney disease, who have type 1 diabetes of more than five years duration or type 2 diabetes mellitus (from the time of diagnosis), and in hypertensive patients to estimate cardiovascular risk and to adjust therapy (Jarraya et al., 2013).

Albumin excretion can be influenced by many extrarenal factors, e.g., fever, exercise, heart failure, poor glycemic control, and inflammatory processes (Bakris, 2001; Gupta et al., 2012). Thus, albuminuria must be interpreted with caution.

6.1.2 Laboratory evaluation of albuminuria

Traditional semiquantitative methods, such as the urinary dipstick and the sulfosalicylic-acid test, are not sensitive and specific enough to estimate urinary albumin levels, although they are more sensitive to albumin than other proteins (Toto, 2004). For a long time, the gold standard measurement of albuminuria in human medicine was assessing the albumin excretion rate (AER) of a 24-hour collection urine sample ("timed urine sample"). In young adults, 5-10 mg/day of albumin excretion is normal and generally rises with age. Over 30 mg/day, there is usually some structural change in the glomerular capillary wall (Levey et al., 2015).

Many studies showed that measuring the urinary albumin-to-creatinine ratio (UAC) of a morning urine sample can give a reliable result of the magnitude of albuminuria (Gansevoort et al., 2005; Ruggenenti, 1998). Thus, in recent recommendations, UAC has been the first-choice method for detecting albuminuria (Levey et al., 2015).

The physiologic value of UAC in people is <30 mg/g (<0.03 g/g). Previously the term microalbuminuria was used to describe low concentrations of albumin in the urine (generally between 30 and 300 mg/g), which is not detectable by the usual colourimetric method on the urinary dipstick. More than 300 mg/g (>0.3 g/g) was considered macroalbuminuria (Toto, 2004). The term microalbuminuria is discouraged in the "Kidney Disease Improving Global Outcomes" (KDIGO) guideline. Instead, a new staging system is recommended (see Table 5) (KDIGO, 2013).

Table 5. A new staging system for albuminuria in human medicine. UAC = urinary albumin-to-creatinine ratio (KDIGO, 2013).

Albuminuria category	UAC (mg/g)	Terms
A1	<30	normal to mildly increased
A2	30-300	moderately increased (formerly "microalbuminuria")
A3	>300 (including nephrotic syndrome, >2200 mg/g)	severely increased (formerly "macroalbuminuria ")

Some authors suggest using race (lower in Caucasians than black people), age (lower in children), and sex-specific (lower in women) cut-off values for UAC because there can be a significant difference between the different groups (Mattix et al., 2002; Miller et al., 2009).

In some cases, UAC is less reliable, e.g., screening for early diabetic kidney disease, evaluating potential kidney transplant donors, or if the patient is not in a steady state (e.g., acute kidney injury). When creatinine excretion is decreased, UAC overestimates albumin excretion, and when GFR is increased, UAC underestimates albumin excretion. In patients with large muscle mass (with increased urinary creatinine excretion), UAC can underestimate AER, while at decreased muscle mass (e.g., amputation), UAC can overestimate AER. In these cases, the AER measurement of a timed urine sample is required (KDIGO, 2013).

In people, the first-morning voided urine sample is recommended for the UAC measurement because it provides lower variability than random samples. It is suggested that the urine sample be stored at 2-8°C until processing. Albumin is stable for seven days at this temperature. Precipitates or cellular components can absorb albumin; thus, centrifugation should remove any cloudiness before storage. Before measurement, the sample must be warmed to room temperature to dissolve residues that might have formed. Freezing can damage albumin; thus, it is not recommended (storage at -20°C resulted in about a 5-20% reduction of albumin). If the sample has to be stored longer, it should be frozen at -70°C (Miller et al., 2009).

Canine urinary albumin was stable after storage for four months at -20°C and after storage for 12 months at -80°C. The same study found no difference in urinary albumin concentrations in cystocentesis and voided canine urine samples (Smets et al., 2010).

Several semiquantitative and quantitative methods are used to detect and measure urinary albumin concentration: immunoturbidimetric, nephelometric, radioimmunoassay, high-performance liquid chromatography, and enzyme-linked immunosorbent assays (Toto, 2004).

UAC can be given in different units: "mg/mmol," "g/mol," "mg/g," or "µg/mg". The KDIGO guideline recommends using the "mg/g" unit (KDIGO 2013; Miller et al., 2009).

In veterinary medicine, UAC is thought to give the most accurate result of albuminuria. However, some studies found that the urinary albumin concentration (UAlb) is also reliable when the urine specific gravity was adjusted to 1010 (Lees et al., 2005; Whittemore et al., 2011).

It is suspected that the physiologic value of albuminuria is <0.01 g/L in dogs and cats in a spot urine sample. The UAlb values between 0.01 and 0.3 g/L are called microalbuminuria (MA) (Harley & Langston, 2012).

In dogs and cats, albuminuria measurement is suggested as a screening test for early renal damage in animals predisposed or suspected to have renal disease (e.g., hereditary nephropathies) and who have hypertension or systemic diseases leading to proteinuria (Harley & Langston, 2012).

Once microalbuminuria is detected, the patient should be monitored closely to evaluate whether it is persistent or transient. If microalbuminuria is persistent, the diagnostic workup is the same as needed for persistent proteinuric patients (Vaden, 2017).

6.1.3 How to measure urinary albumin in dogs and cats?

Several assays are available for detecting albuminuria in dogs and cats: a semiquantitative point-of-care ELISA test and three tests requiring laboratory background: ELISA, immunoturbidimetry, and high-resolution electrophoresis.

The HESKA Early Renal Disease screening (ERD) test for feline and canine albuminuria has been available for many years. It is a semiquantitative in-house test based on ELISA technology, using species-specific monoclonal antibodies. After diluting the urine to a specific gravity of 1010, the test device is inserted into the urine sample and left for at least 3 minutes. The result is based on a subjective interpretation of a colorimetric change. Results can be: "negative" (urine albumin levels are <0.01 g/L), "low positive," "medium positive," "high positive," or "very high positive") (Syme, 2009).

Mardell et al. evaluated the reliability of the ERD test and found a moderately good correlation between UPC and the ERD test. One hundred cats with any kind of disease and 22 healthy cats were involved in this study. 36% of sick cats and 9% of healthy cats were

positive on the ERD test. 13 cats were found positive with the ERD test but had normal UPC. In this case, microalbuminuria could have been an earlier marker of renal damage than UPC and a predictor of future proteinuria, as shown in previous studies (Lees, 2002; Pressler, 2003; Mardell & Sparkes, 2006). Unexpectedly, there were ten cats with elevated UPC but a negative ERD test, which suggested a false-negative ERD test result. However, we cannot conclude without accurately measuring the urinary albumin. The storage of the samples at room temperature and -4 °C did not significantly change the ERD test results. Blood pressure was measured with the Doppler method in 58 cats. There was no correlation between blood pressure and UPC or microalbuminuria. The limitation of this study was that no quantitative measurement of albuminuria was done; only the ERD test was compared to the UPC and the usual urinary dipstick method. Authors state that it is not always easy to distinguish between the categories (e.g., "low positive" or "medium positive") and that the decision is often subjective. Despite this difficulty, they have found a good agreement between two different readers. The repeatability of the ERD test was not acceptable in 23-27% of the cases in cats. This study concluded that the ERD test "should not be relied on as a sole determinant of proteinuria" (Mardell & Sparkes, 2006).

Another study compared the same ERD test with the urinary dipstick, the SSA test, and UPC measurement in 138 canine urine samples. Good correlations were found between all methods, but the UPC correlated best with the ERD test. The limitation of this study was the same; only the urinary total protein was measured and not albumin itself (Garner & Wiedmeyer, 2007).

Pressler et al. tested a human test strip ('Clinitek strip') to detect albuminuria, but it lacked sufficient concordance with the ELISA test (Pressler, 2008).

Because of these issues, using these semiquantitative tests alone is not recommended (Gentilini et al., 2005).

Pressler et al. validated an ELISA assay for measuring urinary albumin concentration (Pressler et al., 2001). Still, as it is a time-consuming method and can hardly be carried out as routine screening, another assay was needed. In 2005, Gentilini et al. validated the immunoturbidimetric (the human gold standard) method for measuring albuminuria in dogs. Immunoturbidimetry is based on the quantitative measurement of agglutination caused by the reaction of albumin and polyclonal anti-albumin antibodies. Although human and dog albumin is 79% homologous, there is only partial cross-reactivity between them. To measure dog albumin accurately, they modified the methodology by calibrating the analyzer with special calibrators. The modified assay was shown to have reliable linearity between 0 and 225 mg/dL, which is adequate for clinical use. The main advantage of this method is that it can be automated and, therefore, less time-consuming (Gentilini et al., 2005).

Murgier et al. compared the accuracy of the ERD semiquantitative test, the immunoturbidimetric assay, and agarose gel electrophoresis to measure albuminuria. They evaluated 307 urine samples of dogs. The immunoturbidimetric assay could measure canine albumin accurately in the range of microalbuminuria (0-30 mg/dL). Electrophoresis gave reliable results of albumin concentrations of >40 mg/dL and overestimated albumin levels in the 0-40 mg/dL range. The ERD test strips had a sensitivity and specificity of 91% and 92%, respectively, for detecting microalbuminuria (at a cut-off level of 1 mg/dL), with a false-negative rate of 9% and a false-positive rate of 8%. The authors stated that the ERD test is useful in the range of microalbuminuria, but many dogs in the "high" (62.5%) and "very high" (90%) groups had overt proteinuria; thus, these fields are unnecessary as albuminuria can be detected by other methods as well (Murgier et al., 2009).

Another study also found a moderate correlation between the ERD test and the immunoturbidimetry method. UAC ratios in the different categories of the ERD test were median 12 (range: 2–131) mg/g for "negative," 79 (44–242) mg/g for "low positive," 325 (164–725) mg/g for "medium positive," 1023 (242–1251) mg/g for "strong positive" results. The sensitivity and specificity of the ERD test were 81% and 88% at the UAC cut-off value of 78 mg/g (calculated as mean ± 2 SD in control dogs). Using the human UAC cut-off value (30 mg/g), sensitivity and specificity were 64% and 100%, respectively (Schellenberg et al., 2008).

Two methods are validated for the measurement of albuminuria in cats. Williams et al. validated a human Particle-Enhanced Turbidimetric Immunoassay for measuring urinary albumin and cystatin C in cats (Williams & Archer, 2016). The other one is a semi-automated high-resolution electrophoretic method with ease and rapid use, high reproducibility and accuracy, and a wide range of linearity. The limit of detection of this test is 1.25 mg/dL, but it is less sensitive under 5 mg/dL than the ELISA test (Ferlizza et al., 2017).

Another study examined whether albuminuria could **differentiate healthy from non-healthy** dogs (Whittemore et al., 2006). Four hundred-eight dogs were included in this study, of which 14% were considered healthy, and the others had infectious, immune-mediated, inflammatory, or neoplastic diseases. The ERD semiquantitative test and immunoturbidimetric measurements showed that the two methods' specificity (ERD: 91.7% vs. 85.4%) and sensitivity (ERD: 36.9% vs. 35.6%) were very similar in distinguishing between healthy and non-healthy dogs. These values are presumably lower than expected because urine samples that were positive on the usual dipstick test were excluded. Both tests showed a significant increase in microalbuminuria with increasing age among healthy dogs, with the presence of any diseases, and with elevated serum creatinine and BUN concentration in some of the disease categories. No associations were found between

albuminuria and sex, blood pressure, fever, pyuria, or bacteriuria. UAC had poor diagnostic usefulness in detecting systemic diseases in this study. This could have been because the cut-off values were too high: 100 mg/g and 200 mg/g. At these values, the specificity of both tests was 100%, but sensitivity was very low: 10% and 3.6 %, respectively (Whittemore et al., 2006).

6.2 Aim of the study

Thus far, no species-specific reference interval (RI) for albuminuria has been established for dogs. Previous studies examining albuminuria in dogs and cats compared small healthy control groups with the various diseased groups.

The aim of our first study was to establish an RI for albuminuria in more than 120 healthy dogs. This number of reference individuals is recommended in order to determine reference limits by nonparametric methods with 90% confidence intervals (Friedrichs, 2012). Our second goal was to define breed-specific reference intervals as well, in case we find significant differences between the albuminuria of different breeds. Sighthounds were a point of interest because these dogs are known to have unique reference values (Zaldívar-López et al., 2011) and Greyhounds were previously shown to excrete more albumin than other breeds (Surman et al., 2012). Breed-specific reference intervals for Beagle dogs could be useful because of their frequent use as laboratory animals.

6.3 Materials and methods

6.3.1 Dog populations

The study included two population groups of dogs. The first group consisted of clinically healthy, client-owned dogs from various breeds and both sexes, from 1 to 12 years of age (n=101). Dogs visiting the Small Animal Clinic of the University of Veterinary Medicine Budapest for their yearly routine health checks were enrolled. Participation in the study was free of charge. All owners signed a consent form ─ approved by the National Scientific Ethical Committee on Animal Experimentation ─ prior to participation.

The second group included clinically healthy laboratory beagle dogs from both sexes, from 1 to 12 years of age (n=23). The beagles were used as healthy controls in another study without receiving any treatment or interventions. The study was approved by the Government Office of Pest County, Department of Food Safety, Animal-, Plant- and Soil Protection (approval No. PEI/001/1708-4/2015). The health check (physical examination,

blood and urine tests) of these dogs was part of the original study; leftover urine samples were used to measure UAC.

Health status was assessed with histories, physical examinations, hematology, serum biochemistry tests, and urinalyses. Dogs with abnormalities on the physical examinations, or the ones that had changes in any of the inflammatory variables (eosinophil/lymphocyte/neutrophil cell count, C-reactive protein, globulins) or renal variables; had major hematologic or biochemical alterations or were microfilaremic were excluded. Dogs with proteinuria (UPC ≥0.2), pyuria (>5 white blood cells/field of view), or hematuria (>5 red blood cells/field of view) were also excluded from the study.

6.3.2 Sample collection and analyte measurements

Experienced personnel performed venipunctures on the cephalic vein with a 21 G needle. Routine haematological (ADVIA 120 [Siemens Healthcare GmbH, Erlangen, Germany]) and biochemical (Beckman Coulter AU480 [Indianapolis, IN, USA]) examinations were completed. For the detection of blood parasites, blood smears were microscopically evaluated as well.

Urine samples were taken mainly by cystocentesis with a 22 or 23 G needle and a 5-10 ml syringe. In some cases, where cystocentesis was not feasible, free-catch urine samples were collected. A complete urinalysis was carried out, including a microscopic evaluation of the urine sediment and the measurement of urinary protein to creatinine ratio (UPC).

Urinary albumin was measured by a chemistry autoanalyzer (Beckman Coulter AU480 [Indianapolis, IN, USA]) using a dedicated reagent kit (OSR6167, microalbumin, immunoturbidimetric). This reagent kit uses monoclonal antibodies against human albumin to generate agglutination to detect urinary albumin concentrations in the range of 0.5 - 30 mg/dL.

Calibration was carried out using pooled canine sera diluted to give desired calibrator concentrations, as described by Gentilini et al:

- “Standard curves were prepared using canine sera collected and pooled from 10 healthy dogs.
- In each case, serum albumin concentration was measured by five replicates.
- The mean was assumed to be the actual canine albumin concentration.
- Serial dilutions of sera were performed in a phosphate-buffered saline solution.
- Dilutions were calculated to obtain expected canine albumin concentrations of 0.50, 1, 5, 13, and 30 mg/dL.

- Ten standard curves with 5 points of calibration were prepared. Each point was assayed in duplicate.
- Coefficients of variation of absorbances at each calibration point between standard curves were calculated.
- The automated analyzer was set at a linearity range between 0.5 and 30 mg/dl and with a 10-fold automated dilution when the albumin concentration exceeded the upper limits" (Gentilini et al., 2005).

Daily quality control was performed using pooled canine sera diluted to desired concentrations in phosphate-buffered saline solution. These solutions were then aliquoted and stored frozen until use. Two levels of quality control solutions were prepared. One at 12 mg/L and another at 142 mg/L. Coefficients of variation for the two levels were 6.24% and 6.08%, respectively.

Urinary creatinine was measured on the same chemistry analyzer with an enzymatic assay (Diagnosticum Kreatinin). Briefly, this assay utilizes creatininase to convert creatinine to creatin and then creatinase to convert creatine to sarcosine. Sarcosine is then reacted by oxygen to generate hydrogen-peroxide with the help of sarcosine-oxidase. The hydrogen-peroxide is then reacted with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidin to give rise to the formation of a red dye which is measured spectrophotometrically at 550 nm wavelength. This reaction is more specific than the most commonly used Jaffé (alkaline picrate) method, as it is less sensitive to the interference from non-creatinine chromogens such as glucose, pyruvate, ascorbic acid, and acetoacetate that can react with picrate (Braun et al., 2003). For this analyte, there is a daily quality control program and a monthly quality assurance scheme in place (Bio-Rad Urine Chemistry and Urine Chemistry Monthly, respectively).

Urinary albumin was normalized to urinary creatinine and was expressed as urinary albumin-to-creatinine ratio (UAC). UAC can be expressed as a unitless value or, more commonly, as albumin mg/g creatinine. It is usually the preference of the laboratory or the publication that determines which one is used (Chavan et al., 2011). In our studies, we followed the human KDIGO guidelines that recommend using the "mg/g" unit (KDIGO, 2013). For urinary total protein, the pyrogallol red reaction was used (Diagnosticum 42051) on the same chemistry analyzer. The same creatinine measurement was used as for the UAC. UPC was measured from one single sample. It is stated by Nabity et al. that a single UPC determination is sufficient for the evaluation of proteinuria status if the UPC is less than 4 (Nabity et al., 2007).

6.3.3 Statistical analysis

Statistical analysis was carried out in "R 4.0.2". As healthy dogs may have UAC values close to zero (or even zero itself), the lower reference limit is irrelevant. Hence, we estimated the 95% upper reference limit (that excludes 5% of the reference population) by the nonparametric method, together with its 90% nonparametric confidence interval.

In accordance with the American Society for Veterinary Clinical Pathology guidelines, as reference individuals were selected randomly from well-defined populations, and their health was confidently established by experienced veterinarians, all reference values were retained, and no outliers were excluded (Friedrichs et al, 2012). The results of Sighthounds and Beagle dogs were analyzed as subgroups as well. The results of the subgroups were compared with the Kruskal-Wallis test.

The UAC results of free-catch and cystocentesis samples were compared with two-sample t-test. The dependence of UAC on age was evaluated by Spearman's rank correlation. Statistical significance was set at $P < 0.05$.

6.4 Results

In total, 164 dogs were enrolled in the study between 2014 and 2021. All dogs lived in Hungary. 40 dogs were excluded because of elevated CRP (n=11), microfilaremia (n=6), eosinophilia (n=6), leukocytosis (n=3), proteinuria (n=10), pyuria (n=1), hyperglobulinemia (n=1), or other biochemical alterations (n=2).

After exclusions the reference population comprised of 124 clinically healthy dogs (data of all dogs are shown in Table S1 [in Chapter 12. Supplementary material]). The breed distribution was as follows: *Hungarian Greyhound* (n=26), *Beagle* (n=23), *mixed breed* (n=26), *Golden Retriever* (n=4), *Border collie* (n=4), *Deutsch Drahthaar* (n=3), *Dachshund* (n=2) *German sheperd* (n=2), *Borzoi* (n=2), *Dogo Argentino* (n=2), *Australian kelpie* (n=2), *Belgian Malinois* (n=2), *Hungarian vizsla* (n=2), *Miniature Pinscher* (n=2), *Collie* (n=2), *Akita* (n=2), *Labrador Retriever* (n=2). 16 other breeds were represented with one dog in each group.

Dogs ranged in age from 1.0 to 10.7 years (median 3.6 years). There were 81 female (20 spayed) and 43 male (11 castrated) dogs within the population. The weight range was 5.0-43.0 kg (median 16.6 kg).

81/124 (65.3%) urine samples were collected by cystocentesis and 43/124 (34.7%) were free-catch samples. There was no difference between the UAC results of these two groups (p=0.12).

6.4.1 Reference intervals

Using the nonparametric method, a reference interval for UAC of 0—19 mg/g (0 to 0.019) was obtained. The 90% confidence interval for the upper limit for UAC was 13—28 mg/g (0.013—0.028). The median value was 3.0 mg/g (0.003) with a total range of 0 to 48 mg/g (0 to 0.048) in the reference population.

Data of all dogs, Sighthound, and Beagle subgroups are shown in Table 6. No significant differences were found in the UAC values between Beagles, Sighthounds, and the rest of the dogs by the Kruskal-Wallis test (p=0.92).

As the subgroups consisted of <40 dogs, and the UAC did not differ in the different breeds, no reference intervals are given for the subgroups. Histograms, mean, median, minimum, and maximum values are given (Figure 8 and Table 6).

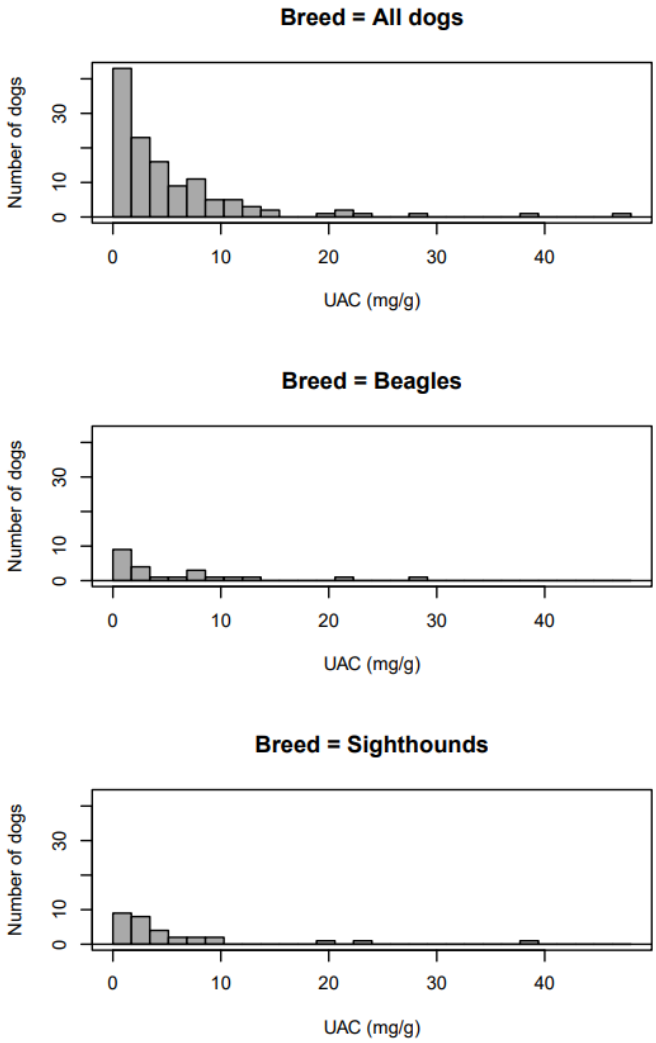

Figure 8. Distribution of UAC in the study population and in the Sighthound and Beagle subgroups.

Table 6. Summary of population characteristics, results of urinary albumin concentration (UAlb), urinary protein-to-creatinine ratio (UPC), urinary albumin to creatinine ratio (UAC) and reference intervals (RIs) for UAC with 90% confidence intervals (CIs). As the subgroups consisted of <40 dogs, and the UAC did not differ in the different breeds, no RIs and CIs are given for the subgroups (N/A=not applicable).

	All dogs	Beagles	Sighthounds
Number of dogs	124	23	30
Age (years)	4.1±2.1	2.4±0.7	4.6±2.0
BW (kg)	18.9±10.0	10.6±1.5	25.9±6.2
UAlb (mg/L)			
Mean	11.5	6.1	18.0
Median	7.0	5.0	11.4
Range (min; max)	0; 108	0; 46	1; 108
UAC (mg/g)			
Mean	5.2	5.8	5.6
Median	3.0	3.0	3.0
Range (min; max)	0; 48	0; 28	0; 39
Reference interval	0-19	N/A	N/A
Lower Limit 90% CI	0-0	N/A	N/A
Upper Limit 90% CI	13-28	N/A	N/A
UPC (mg/g)			
Mean	0.08	0.11	0.06
Median	0.07	0.1	0.05
Range (min; max)	0.02; 0.19	0.05; 0.19	0.02; 0.16

The dependence of UAC on age was not significant (Spearman's rho = 0.149, p=0.098) (Figure 9). No significant differences were found in the UAC values between the various bodyweight and sex groups (Figure 10).

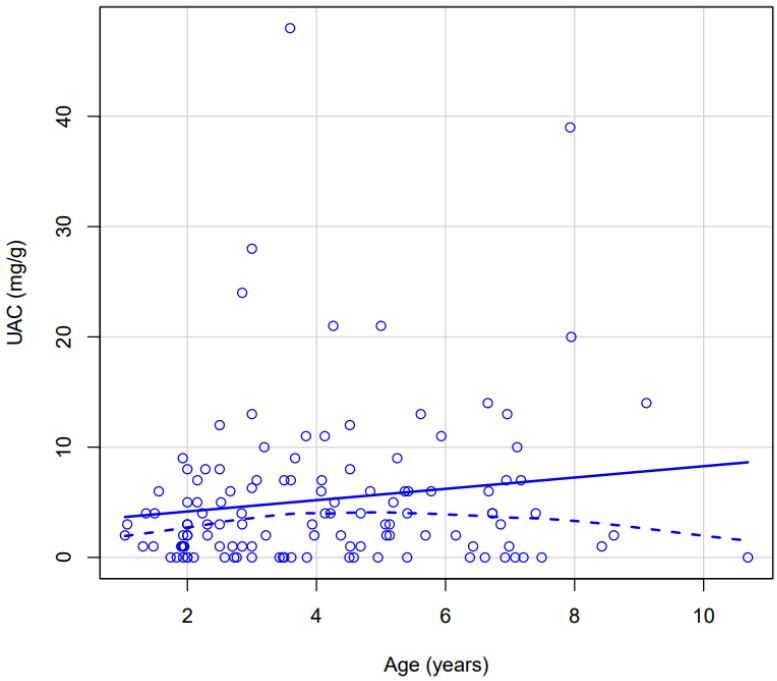

Figure 9. Scatterplot showing the distribution of UAC by age. Each symbol 'o' indicates the result of each individual dog.

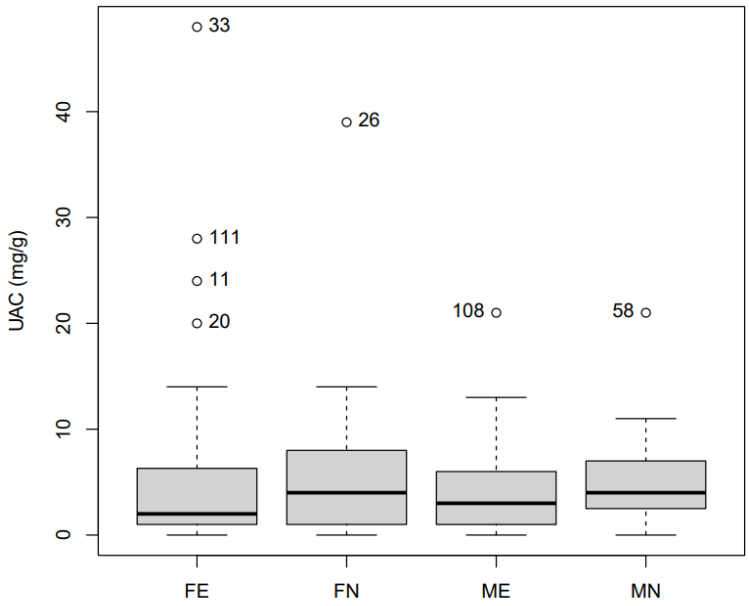

Figure 10. Boxplot of canine urinary albumin to creatinine ratio (UAC) in the different sex groups. The box represents the interquartile range and is bisected by a line representing the median. The dashed lines represent the range of data with open circles representing outlying points. FE= female entire; FN=female neutered; ME=male entire; MN=male neutered

6.5 Discussion

Our study is the first to describe UAC in a dog sample of more than 120 dogs that is required for establishing a reference interval according to the American Society for Veterinary Clinical Pathology guidelines (Friedrichs et al., 2012). Prior to this study, there have been a limited number of reports describing the UAC in smaller numbers of healthy dogs (Mazzi et al., 2008; Smets et al. 2010; Bacic et al., 2010; Schaefer et al., 2011; Schellenberg et al., 2008). The UAC values of healthy control groups from prior studies are shown in Table 7.

Table 7. The urinary albumin-to-creatinin ratios (UAC) of healthy dogs in different studies.

Author, year	Nr. of healthy animals	UAC (mg/g) median (range) except where stated	Method
Mazzi, 2008	21	20 ± 70 (mean ±SD)	immunoturbidimetry
Schellenberg, 2008	6 and 6 in two different groups	17 (5–82) and 15 (9–23)	immunoturbidimetry
Smets, 2010	10 young dogs: age 2.4 (1.1–2.9) years	4.7 (1.5– 46.3)	ELISA
Smets, 2010	10 older dogs: age 8.3 (7–10.9)	17.8 (3.3–296.5)	
Bacic, 2010	39	2 (0.5–10)	ELISA
Schaefer, 2011	15	1.9 (0.2–8.3)	ELISA
Tefft, 2014	20 ideal bodyweight	0.18 (0–7.04)	immunoturbidimetry
Tefft, 2014	22 overweight/obese	0.41 (0–10.39)	immunoturbidimetry

The median (range) UAC of 3 (0; 48) mg/g found in our study is similar to those previously reported (Table 7).

The reference interval for canine UAC was 0 – 19 mg/g in a clinically healthy dog population. The human reference interval is somewhat higher: 0 – 30 mg/g, but very similar to the upper limit 90% confidence interval of 13–28 mg/g, found in this study.

In this study, we used one-sided reference intervals. This statistical method is recommended in cases when one tail of the reference distribution is clinically irrelevant. In our case, for example, UAC values near zero, or zero itself may well occur in healthy dogs. Some authors point out that in such cases it is reasonable to consider the 95% percentile instead of the 97.5% percentile as the upper cutoff value (Koduah et al., 2004; Boyd, 2010).

We did not exclude any outliers in our study. The American Society for Veterinary Clinical Pathology guidelines state that outlier tests require Gaussian distribution of data, or at least that data are transformable to Gaussian (Friedrichs et al., 2012), which is not the case with UAC. This distribution is very skewed, with so many values near zero that it cannot be transformed to Gaussian. In such cases removing the largest value(s) may cause a rather strong bias. According to the American Society for Veterinary Clinical Pathology guidelines, if a nonparametric reference interval is applied (as in our case), outliers can make less trouble "because nonparametric methods establish reference limits by trimming the most extreme values, outliers have less of an effect on the reference interval than with parametric and robust methods" (Boyd, 2010). Without any prior knowledge about the distribution of UAC values in healthy dogs, there is no reason to claim that UAC values of some dogs are outliers.

Visual inspection of Figures 10 and 12 may suggest that the highest values are outliers but the guideline warns that exclusion must be justified because "these values may represent the true distribution of values in a healthy population" (Friedrichs et al., 2012).

In our study, the UAC values of 5/124 (4%) dogs were over 19 mg/g. Their result was: 20, 21, 28, 39, and 48 mg/g. We presume that there is a "borderline" range for albuminuria as well (somewhere between 20-50 mg/g), just like for proteinuria (UPC 0.2-0.5), which is a gray zone and includes transiently proteinuric but otherwise healthy dogs and dogs with pathologic proteinuria as well. We assume that these five dogs were healthy, and this mild albuminuria represents a harmless laboratory change that can be present anytime because of, e.g., subclinical inflammation or endothelial cell damage or dysfunction. Two dogs with the highest UAC (39 and 48 mg/g) were followed. Both dogs were still alive five years after sample collection, without any renal involvement. These two dogs also prove that albuminuria is not a specific marker for renal disease, but it can be a transient and harmless laboratory change.

Two large-scale studies on healthy dogs (n=3041) and cats (n=611) found that albuminuria increases with age (using the ERD semiquantitative test) (Langston, 2004; Radecki, 2003). Almost one-fourth of all dogs tested positive; 4% of the one-year-old dogs and 55% of the 15-year-old or older animals (Radecki, 2003). Concerning healthy cats, the overall prevalence of albuminuria was 14%, which also increased with age. In the age group of 12-15 years, the prevalence was more than 30%, and between 16-23 years, the prevalence was more than 70% (Langston, 2004). Our study could not confirm this; there was no difference between the UAC values in the different age groups. This discrepancy could be because we used more stringent exclusion criteria: dogs with any change in any inflammatory or renal variables (more frequent as dogs age) were excluded. When the microalbuminuria-positive dogs from the study of Radecki et al. were followed, 56% were diagnosed with inflammatory, neoplastic, or metabolic diseases, 31% had renal disease, and 12% had no specific diagnosis (Whittemore et al., 2006).

Surman et al. examined clinically healthy retired racing greyhounds. The semiquantitative ERD test found microalbuminuria in 53% of the dogs. Twenty-eight percent was "low positive," 21% "medium positive," and 4% "high positive." Sixty-two percent of dogs were considered hypertensive (systolic BP >160 Hgmm). There was a positive correlation between hypertension and microalbuminuria; 82% of hypertensive dogs had microalbuminuria, while only 18% of normotensive dogs showed microalbuminuria. Renal biopsies were also taken in 15 cases for light microscopic, electron microscopic, and immunofluorescent evaluation. Many dogs had mild nonspecific histopathologic changes, e.g., all 15 dogs had podocyte effacement, and 13/15 dogs had diffuse staining for IgM. Still, no associations were found between the biopsy scores and microalbuminuria or hypertension (Surman et al., 2012). Our study could not confirm that Sighthounds have more frequent albuminuria than other breeds. Three reasons could explain the difference. First, it is possible that the exclusion criteria in the previous study were not as strict as ours. Second, they used a different method to estimate albuminuria (a semiquantitative method), and third, our Sighthound populations consisted mainly of *Hungarian Greyhounds*, which are supposedly genetically different from *(English) Greyhounds*. We also measured blood pressure in the Sighthound group. In our study, none of them had hypertension, while in the previous study, 62% of the Sighthounds had hypertension. There could be many reasons for this difference. They used the Doppler and the oscillometric method, while we used the High Definition Oscillometric method. Their dogs were hospitalized for a veterinary student spay and neuter program while we did our measurements with the calming presence of dog owners. Furthermore, again, *Hungarian Greyhounds* can differ from *(English) Greyhounds.*

Another study also suggested that the likelihood of albuminuria may be breed-dependent. *Labrador* and *Golden retrievers* were found to have a higher odds ratio for albuminuria than mix-breed dogs (Radecki et al., 2003). Our study found no difference between the different breeds, although only 4 *Golden Retrievers* and 2 *Labrador retrievers* were represented.

Tefft et al. compared albuminuria and proteinuria in 22 obese dogs with 20 ideal body weight controls. The two groups had no significant difference (Tefft et al., 2014). Based on their findings, obesity was not an exclusion criterion in our study. As most of the examined dogs had responsible owners, most had normal to mildly increased body condition scores in our research. In our study, smaller-sized and bigger-sized dogs (with more significant muscle mass) were also represented, and we did not detect any extreme changes in muscle mass (e.g., cachexia or missing limb). Thus, the urinary creatinine results also represent a healthy dog population and did not distort our results.

One limitation of the study was that UPC was not determined in 35 dogs (not within the Beagle or Sighthound subgroups). However, as UAC values were low in all of these dogs (ranged from 1 to 14 mg/g), we presumed that these dogs would not have had a high UPC (hence would not have been excluded), thus including these dogs was reasonable.

Another limitation was that for the detection of *Dirofilaria* species only blood smear evaluation was carried out in all dogs. Heartworm antigen test and Knott-test (for the detection of microfilariae) were only done in the Beagle subgroup. Although it was not an inclusion criterion, most of the examined dogs received monthly prevention against *Dirofilaria* species.

Blood pressure was only measured in Sighthounds and not registered in the other subgroups. However, hypertension in dogs develops mostly secondary to chronic illnesses (e.g. kidney disease, endocrine diseases) (Acierno et al., 2018). As we excluded all dogs with suspicion for concurrent illnesses, it is very unlikely that dogs with subclinical hypertension were included in our final reference population.

In conclusion, the reference interval for urinary albumin to creatinine ratio of 0 ─ 19 mg/g was established from 124 healthy dogs. Breed, age, sex, bodyweight or collection method does not seem to influence UAC. Our study included a diverse population, with representation from thirty-two different breeds and numerous mix-breed dogs. This diversity in the study population adds depth to our findings and underscores the potential applicability of our results across a wide range of dog breeds and types.

7. Albuminuria and proteinuria in dogs infected with *Dirofilaria repens*: a cross-sectional study

7.1 Overview of *Dirofilaria repens* infection of dogs

7.1.1 Background

Dirofilariasis is a nematode infection of domestic and wild canids transmitted by mosquito vectors. Dirofilarias belong to the Nematoda phylum, Spirurida order, Filariodea superfamily, and Onchocercidae family. The two most important species within the *Dirofilaria* genus are *D. immitis* and *D. (Nochtiella) repens*. *D. immitis* is the causative agent of canine and feline heartworm disease, while *D. repens* causes subcutaneous and ocular dirofilariasis in canids (Kassai, 2011). While the first case of *D. immitis* was found in the 17th century in a hunting dog (Simón et al., 2012), *D. repens* was only described and named in 1911 by Railliet et Henry (Capelli et al., 2018).

Both infections are considered emerging zoonoses with public health concerns worldwide (Simón et al., 2012).

7.1.2 Prevalence

D. immitis is dispersed worldwide in tropical and temperate regions, while *D. repens* is only present in the Old World. Both species are detected with increasing frequency due to climate change and the appearance of new, invasive, and competent mosquito species (e.g., Aedes albopictus and Ae. koreicus) (Genchi & Kramer, 2020).

Autochtonous *D. repens* infection has already been described in most European countries, in many countries in the Middle and Far East, and in some African countries. The prevalence of *D. repens* in dogs is high in the Mediterranean countries, such as Italy (1.5-12%), France (8.5%), northern Greece (up to 30%), and, in some central and eastern European countries too, such as Poland (11.5%), Hungary (18.1%), Croatia (14-47.3%), Serbia (17-49%), Slovakia (34.5%), and Russia (10-43%) (Capelli et al., 2018).

Human dirofilariasis was first detected in Hungary in 1951 (Kotlán, 1951). The first description of *D. repens* in Hungary in a dog dates back to 1998. The prevalence of *D. repens* was surveyed in Hungary between 2005 and 2009 by Jacsó et al. Researchers found a prevalence of 18.1% in dogs and 3.8% in cats. In some areas, the prevalence of dogs was over 25% (Jacsó, 2014). A recent Hungarian survey found that 11.1% of the examined dogs

were infected with *D. repens* and 8.1% with *D. immitis*. Coinfections were detected in 3.2% of the dogs (Farkas et al., 2020).

7.1.3 Life cycle

D. repens can be transmitted by more than 60 mosquito species, such as *Anopheles, Aedes, Culex*, and *Coquillettidia* species (Capelli et al., 2018; Demiaszkiewicz, 2014). Dogs and other carnivores (e.g., wolves, foxes, coyotes, jackals) are the definitive hosts. However, as the parasite shows poor vertebrate host specificity, other mammalians (e.g., felids) and humans can also get infected. Domestic and wild dogs function as reservoirs (Capelli et al., 2018).

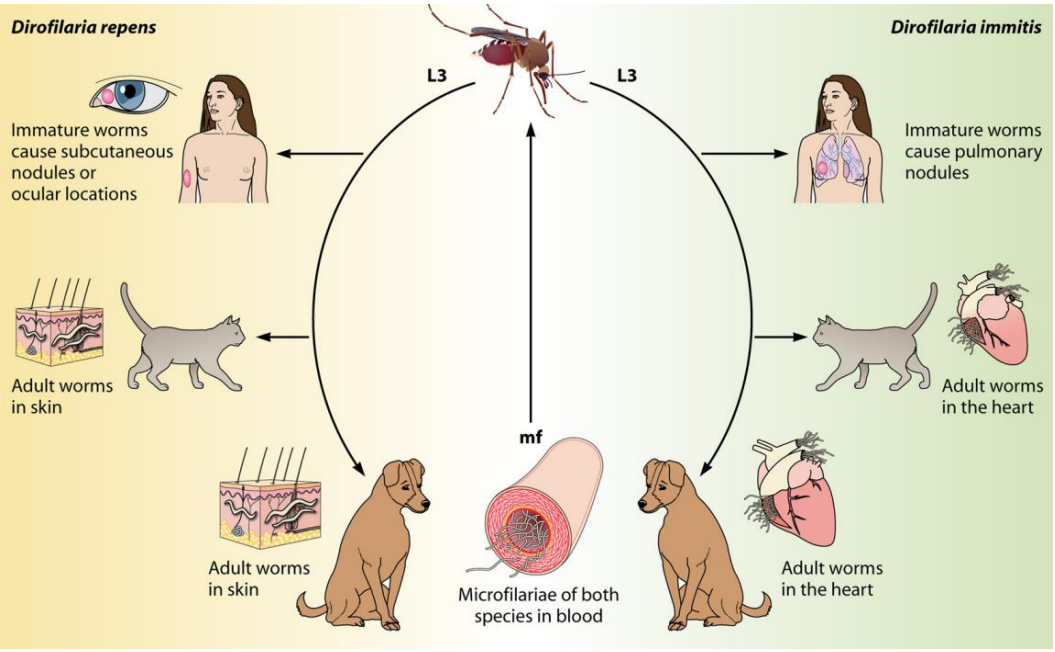

Figure 11. Biological life cycle of *D. immitis* and *D. repens*. Mf = microfilaria (Simón et al., 2012)

The mosquito vector ingests the microfilariae during a blood meal, then they molt through the L1-L2 stage larvae to the infective L3 larvae within 8-20 days (Figure 11). This process is temperature-dependent and is slower at lower temperatures. The L3 larvae penetrate the mosquito's mouthparts and wait until they get transmitted. During the next blood meal, the L3 larvae migrate to the surface of the host's skin and then penetrate the subcutaneous tissue on their own through the wound. The L3 larvae undergo two additional molts (to L4 and preadult forms) and then mature into adult worms in the host's

subcutaneous tissue. Dirofilarias are viviparous worms, and microfilariae are released into the bloodstream after mating. The prepatent period is 6-9 months (Capelli et al., 2018). The production of microfilariae can last up to 3 years. Females can produce up to 5.000 microfilariae per day (Tarello, 2011).

The adult worms generally stay in the subcutaneous (or intramuscular) tissue, but they can sometimes be found in the abdominal cavity or the scrotum. The female nematodes (which grow bigger than males) can reach 10-17 cm (Simón et al., 2012). The worms live 2 to 4 years on average but may live up to 10 years (Capelli et al., 2018).

Both *D. repens* and *D. immitis* harbor an endosymbiont bacterium, *Wolbachia pipientis*. It is essential for the long-term survival of the dirofilarias and their reproduction. It produces pro-inflammatory and chemotactic cytokines and has a significant role in desensitizing the host's immune system (Capelli et al., 2018).

7.1.4 Clinical presentation

D. repens infection is usually associated with no or only minor clinical signs in dogs. The worms can be found in the subcutaneous tissue, sometimes in subcutaneous nodules, the conjunctiva, or the scrotum (Simón et al., 2012). If symptoms are present, they are most dermatologic: pruritus, erythema, dermal swelling, subcutaneous nodules, (circular) alopecia, pyoderma, lichenification, hyperpigmentation (Genchi & Kramer, 2017). The subcutaneous nodules are generally cold, not painful, and mobile without showing inflammatory reactions. Ocular lesions (e.g., conjunctivitis) happen rarely (Capelli et al., 2018). Dermatologic signs are caused by capillary embolization of microfilariae, movement of adult worms in the subcutaneous tissues, immunological and allergic reactions to the parasites, and toxins released by the worms (Tarello, 2011).

In a 435 *D. repens* infected dogs survey, 18.4% showed dermatologic signs (Jacsó, 2014). In another survey, 100% of the 100 examined dogs showed dermatologic symptoms: pruritus (100%), erythema (79%), papulae (62%), focal or multifocal alopecia (55%), hyperkeratosis (18%), crusting (14%), nodules (12%), acanthosis (5%), eczema (3%), pyoderma (3%), and edema (1%) (Tarello, 2011). Skin lesions usually recur seasonally from spring to autumn during the second and third years of infection and become persistent after the fourth year. The more pronounced immune stimulation and toxic effect are caused by the increased number of microfilaria, adult worms, and reinfection (Tarello, 2011).

Extra dermal signs are extremely rare and are primarily associated with massive worm burden: lymphadenomegaly, anorexia, vomiting, fever, and lethargy (Simón, 2012). In a survey of 100 *D. repens* infected dogs, the extra dermal signs were as follows: conjunctivitis

(46%), anorexia (35%), vomiting (26%), fever (25%), lethargy (20%), and lymphadenomegaly (10%). Systemic signs are primarily associated with some concurrent underlying diseases or infective agents and are presumably not caused by *D. repens*. Coinfections with *Ehrlichia* spp., *Hepatozoon canis*, *Leishmania* spp., *Babesia* spp., and *Anaplasma phagocytophilum* have been described (Tarelli, 2011).

Few pathology reports described gross and histopathological changes in many organs (spleen, liver, kidneys, lungs, heart, brain) in massively infected dogs. However, these dogs also had different comorbidities (Grandi, 2007).

7.1.5 Diagnosis

In Europe, where *D. repens* and *D. immitis* are simultaneously present, distinguishing between these two species is crucial, as it makes a huge difference concerning therapy and prognosis (Capelli et al., 2018). In some European countries, a third filarial parasite, *Acanthocheilonema* spp., can also be present (Pacifico et al., 2021).

Adult *D. repens* nematodes can be morphologically identified when found in skin lesions, during surgeries, or on histopathological examinations. On gross evaluation, *D. repens* adults appear with whitish cuticles, distinct longitudinal ridges, and narrows at the end. Males are smaller: 48-70 mm x 3.7-4.5 mm, while females can reach 100-170 mm x 4.6-6.5 mm in size (Capelli et al., 2018).

The diagnosis is often made by a blood test. Sometimes, microfilariae can be found on a direct blood smear, but the modified Knott's test is generally used in the clinical field to detect the larvae (Capelli et al., 2018). Microfilariae show periodicity; we can find the highest number in circulation in the evening (Di Cesare et al., 2013; Ionică et al., 2017), although the reduction in their number is only about 20-40% around noon. Hence, taking the blood sample any time during the day would not risk false-negative results (Tarello, 2011). To distinguish between the different species, microscopic evaluation of the microfilariae, histochemical staining (e.g., acid-phosphatase activity), or molecular biologic tests (conventional and real-time polymerase chain reaction [PCR], probe-based or high-resolution melting analysis techniques) can be performed. In the routine diagnostic workup of these patients, the PCR test is essential for the diagnosis. *D. repens* microfilariae have an obtuse-rounded cephalic margin and a long, often curved tail, and they do not have a sheath (Capelli et al., 2018). In a study, researchers could distinguish between *D. repens*, *D. immitis,* and *Acanthocheilonema spp.* based on their size. The *D. repens* larvae were the biggest (369.44 ± 10.76 µm x 8.87 ± 0.58 µm), while *D. immitis* larvae were smaller, and *Acanthocheilonema spp*. were the

smallest (Magnis, 2013). The real-time PCR technique has a very high sensitivity, and a minimal amount (0.3 pg/ml) of DNA is enough to detect microfilariae.

In-clinic antigen tests are only available for *D. immitis*, not *D. repens*. A positive Knott's test with a negative antigen test suggests *D. repens*, although a false-negative *D. immitis* antigen test is possible in case of low worm burden. Due to the nature of the infection, the earliest time when heartworm antigens and microfilariae can be detected is about 5 to 6 months after infection. Thus, there is no reason to test a dog for *Dirofilaria* infection before six months of age (American Heartworm Society, 2024).

7.1.6 Prevention

To prevent infections from both *Dirofilaria* species, macrocyclic lactones (ivermectin, milbemycin-oxime, moxidectin, and selamectin), given according to label instructions, are recommended. The prevention should be started latest at eight weeks of age. Puppies started on a macrocyclic lactone later than eight weeks of age should be tested for heartworm infection six months after the initial dose and annually after that. In dogs over seven months of age, antigen and microfilaria testing should be done before starting a preventive regimen (American Heartworm Society, 2024).

Previously, some authors suggested that in areas where mosquitos are not present in winter, prophylaxis must start one month before the transmission period and stop one month after this period has ended (Simón et al., 2012). The American Heartworm Society and the European Society of Dirofilariosis and Angyostrongylosis recommend continuous annual prophylaxis administration (American Heartworm Society, 2024; ESDA, 2017).

Repellent agents and ectoparasiticides are also essential parts of prevention. Many repellent products are registered against *Culex* and *Aedes* mosquitoes; most contain pyrethroids. Isoxazoline ectoparasiticides (e.g., sarolaner, fluralaner, afoxolaner) kill mosquitos within 1 to 3 days after they come into contact with the treated dogs' blood, and they prevent egg laying of the female mosquito. L1 larvae cannot molt into the infective L3 within this short period. Thus, it prevents the transmission of the infection. Although repellent agents and ectoparasiticides are beneficial in preventing dirofilariasis, their use alone or together is insufficient (American Heartworm Society, 2024).

7.1.7 Treatment

The treatment of *D. repens* is relatively simple compared to the complexity of treating *D. immitis*. One single dose of moxidectin+imidacloprid spot-on preparation was enough to eliminate all the D. repens microfilariae from the circulation (Frangipane di Regalbono et al., 2016), and six consecutive monthly treatments could also eliminate all the adult D. repens worms (Petry, 2015).

7.1.8 Glomerular dagame caused by *D. immitis*

It has long been known that *D. immitis* can cause glomerular damage. Many studies described (mostly membranoproliferative) glomerulonephritis in dogs with spontaneous and experimentally induced heartworm infection (Simpson et al., 1974; Casey & Splitter, 1975; Shirota et al., 1979; Abramowsky et al., 1981; Grauer et al., 1987, Paes-de-Almeida et al., 2003). The most commonly described ultrastructural lesions are thickening and vacuolization of the glomerular basement membrane (GBM), mesangial cell proliferation, and foot process effacement. Electron-dense deposits were frequently found in the GBM and mesangium. Although glomerular lesions can be pronounced, kidney failure and azotemia are usually absent (Paes-de-Almeida et al., 2003).

Adult heartworm antigens are believed to cause kidney damage, but the presence of microfilariae can exacerbate the condition (Morchón, 2012; Hormaeche, 2014).

A study confirmed in situ immune complex formations in experimentally infected dogs. They found that only 14 days after injection of *D. immitis* antigens into the left renal artery, all dogs (8 out of 8) developed glomerular lesions, and 7 out of 8 dogs showed a positive reaction for IgG on immunofluorescence examination. 3 out of 8 dogs also developed lesions on the right kidney (Grauer, 1989). Another study measured the concentration of circulating immune complexes (CIC) in 34 infected dogs and found that CIC levels correlated with the adult worm burden but not with the number of circulating microfilariae (Nakagaki, 1990).

Another study examined 16 beagle dogs experimentally infected with *D. immitis* and highlighted the role of microfilariae. The most severe glomerular lesions were found in dogs with high microfilarial counts and more than one year of duration. Only mesangial changes were visible when dogs were examined after a short infection period (111 days) when they did not have microfilariae yet (Paes-de-Almeida et al., 2003).

Two studies found that microfilaremic dogs have more severe proteinuria than those without (Morchón, 2012; Hormaeche, 2014). Morchón et al. found only mild histopathologic alterations in the kidneys of microfilaria-negative dogs, whereas typical changes were observed in microfilaria-positive dogs. Heartworm-infected dogs had significantly higher

levels of urinary anti-Wolbachia-Surface-Protein antibodies than controls, and dogs with circulating microfilaria had higher levels of antibodies than those without. They also found in the kidneys in situ production of specific IgG against *Wolbachia* bacteria and concluded that Wolcbachia also plays a role in the pathogenesis of glomerulonephritis (Morchón, 2012).

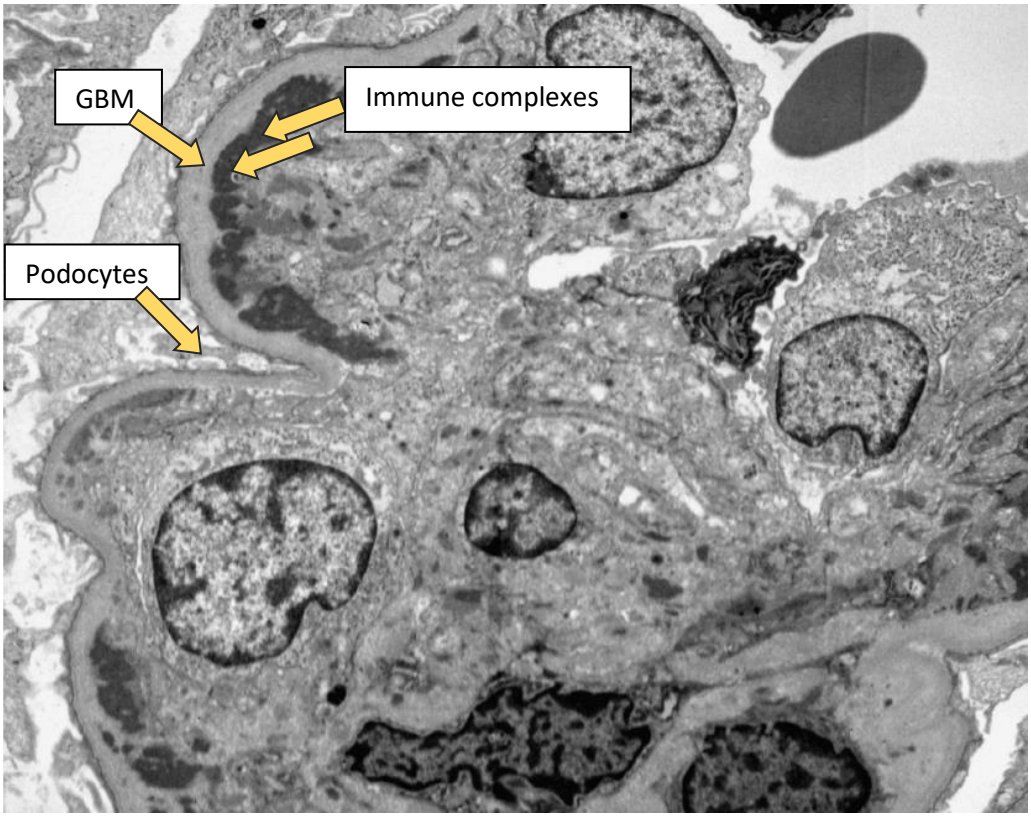

Figure 12. Transmission electron microscopic picture of a glomerulus with membranoproliferative glomerulonephritis. "There are subendothelial and mesangial electron-dense deposits (red arrows). This segment of the glomerular tuft is hypercellular." GBM = glomerular basement membrane (Cianciolo et al., 2018).

7.1.9 Possible renal dagame caused by *D. repens*

During her Ph.D. studies, dr. Olga Jacsó raised the awareness of potential kidney damage caused by *D. repens* infection. She found significantly increased serum urea values in the infected dogs. In the same dissertation she described two cases where the pathologic examination showed renal fibrosis in one dog and membranous glomerulonephritis in another, infected dogs with *D. repens*, although the cause-and-effect relationship was questionable (Jacsó, 2014).

A study from Poland also described two necropsy reports in which dogs infected with D*. repens* had renal changes: membranoproliferative glomerulonephritis, focal glomerulosclerosis, and interstitial inflammation (Osińska, 2014).

Mircean et al. described a case of a five-year-old Boxer dog that was euthanized because of end-stage kidney disease and coinfection of *D. repens* and *D. immitis*. The histopathology examination showed membranoproliferative glomerulonephritis and intralesional microfilariae. Microfilariae were present not only in the renal vessels but also in the tubular lumen and the interstitium. A PCR test was run on the renal biopsy specimen, and it showed the presence of only *D. repens*, not *D. immitis. D*. *repens* microfilariae were also present in the dog's urine sample, along with severe proteinuria. The authors assumed that renal damage was mainly caused by *D. repens* (Mircean, 2017).

Although these case studies suggest possible renal damage caused by *D. repens,* there is still little evidence.

7.2 Aim of the study

The aim of our second study was to investigate, whether *D. repens* infected dogs have a higher magnitude of proteinuria or albuminuria than non-infected dogs, kept under the same circumstances. Our study also aimed to investigate, whether the magnitude of protein or albuminuria would decrease after eliminating the infection. In addition to the primary and secondary goals of our study, we compared some other laboratory variables (hematology, serum urea, and creatinine, urine specific gravity) between the infected and non-infected dogs. Our third goal was to evaluate UAC and UPC changes after topical moxidectin treatment.

7.3 Materials and methods

7.3.1 Animals

This cross-sectional designed study was performed on laboratory beagle dogs. 65 clinically healthy laboratory beagle dogs from 1 to 10 years of age were recruited between 2014 and 2016. The dogs were bred for experimental purposes and were housed in two institutions (authorized by the Government Office of Pest County, Department of Food Chain Safety, Animal-, Plant- and Soil Protection). Animals were cared for according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. Dogs were housed in groups of 4-5 in pens with an outdoor run possibility. The animals were provided with drinking water ad libitum and were given dry food once a day. Animals were vaccinated yearly and dewormed with praziquantel, pyrantel pamoate, and fenbendazole every 3 months. None of the dogs received macrocyclic lactone products as prevention.

The health status of the dogs was assessed during their yearly routine health screening with complete physical examinations, hematology, and serum biochemistry tests, along with urinalyses.

In this study, dogs were tested for *D. repens* infection and were grouped as "infected" or "control" dogs.

Dogs with abnormalities on the physical examination or the ones that had elevated C-reactive protein (CRP) concentration, white blood cell count (WBC), or abnormal renal function, as well as those with major hematologic or biochemical alterations, were excluded. We also excluded dogs with any suspicion of *D. immitis*, *Ehrlichia canis*, *Anaplasma phagocytophylum*, or *Borrelia sensu lato* infection. Dogs with pyuria or hematuria and dogs with positive urine culture examination results were excluded from the study.

The study was approved by the Government Office of Pest County, Department of Food Chain Safety, Animal-, Plant- and Soil Protection (approval No. PEI/001/1708-4/2015).

All *D. repens* infected dogs received appropriate treatment against the infection after the examinations (topical moxidectin/imidacloprid therapy, according to the instructions of the manufacturer: 25 mg for dogs between 4-10 kg and 62.5 mg for dogs between 10-25 kg, every 4 weeks, for 6 months).

7.3.2 Sample collection and analyte measurements

Experienced personnel performed venipunctures on the cephalic vein with a 21 G needle. Routine haematological (ADVIA 120 [Siemens Healthcare GmbH, Erlangen, Germany]) and biochemical (Beckman Coulter AU480 [Indianapolis, IN, USA]) examinations

were completed. For the detection of blood parasites, blood smears were microscopically evaluated.

For the detection of *D. immitis* and *D. repens* microfilariae, the modified Knott's test was performed. One milliliter of EDTA anticoagulated blood was mixed with 9 ml distilled water in a 10 ml conical centrifuge tube. The mix was agitated for 30 minutes on a hematology blood sample mixer. The tubes were then centrifuged at 4000 revolutions per minute for 10 minutes. The supernatant was decanted, and the sediment was stained with new methylene blue. Wet mount slide preparations were examined under the microscope for microfilariae.

As the magnitude of microfilaremia can fluctuate (Ionică et al., 2017), at those dogs where it was feasible, a pre-screening Knott's test was run a few weeks before the examinations, to assess the magnitude of *Dirofilaria* infection of the colony and to make sure not to miss any infected dogs. At the start of our cross-sectional examinations, Knott's test was done on all dogs irrespective of the pre-screening test results.

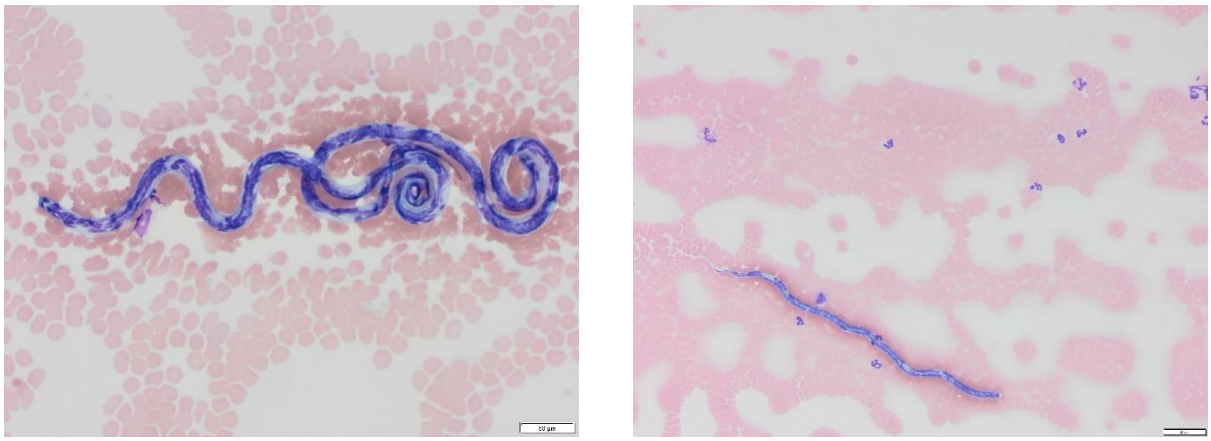

Figure 13. *D.repens* microfilaria (courtesy of Praxislab Kft.)

For dogs with a positive Knott's test, a PCR test was run to differentiate between *D. repens* and *D. immitis* infection. DNA was extracted from EDTA blood samples using the QIAGEN QIAamp DNA Mini KIT according to the manufacturer's instructions. In some cases, the extraction was carried out on a QIAGEN QIAcube semi-automated DNA extraction analyzer. The proprietary qPCR test mix contained *D. immitis* of specific primers (22 & 25 nt), and probe (27 nt, 5'-FAM, 3'-TAMRA) targeting a 302 base pair (bp) long region of internal transcribed spacer 2 (ITS2) region of the 5.8S ribosomal RNA gene. It also contained *D. repens* specific primers (both 24 nt), and probe (30 nt, 5'-TxRd, 3'-BHQ2) targeting a 209 bp long region of cytochrome c oxidase subunit I (COX1) gene. The reaction mixes of 10 µl per tube contained 300/100 nM of the *D. immitis* primer and probe and 200/100 nM of the *D. repens* primer and probe, using the Fast Start Essential DNA Probes Master (Roche) kit

reagents according to the manufacturer's recommendation. The qPCR was performed and read in a LightCycler © 96 thermocycler machine with the following conditions. Preincubation at 95 °C for 10 min followed by 45 cycles of a three-step amplification: at 95 °C for 10 sec, 55 °C for 15 sec, and 60 °C for 45 sec. DNA isolated from adult *D. repens* worms was used as positive control and DNA-free distilled water was used as negative control.

Dogs were considered as "*D. repens* infected" if they were microfilaremic and their PCR test verified *D. repens* infection, but not *D.immitis* infection and both of their *D. immitis* antigen tests were negative. Dogs without microfilaremia (both Knott's tests were negative) and with two negative *D. immitis* antigen tests were used as "control" dogs.

For the detection of *D. immitis* antigens, we used two different tests for all dogs. The Dirocheck ELISA kit (Synbiotics, US, lately Zoetis) was used on an automated ELISA analyzer (Personal-Lab, Italy or Siemens BEP 2000, Germany). In each run, positive and negative control sera (provided by the manufacturer within the test kit) were tested alongside the study samples. The other test used was the SNAP 4Dx Plus Test (IDEXX Laboratories, Inc.) according to the manufacturer's instructions.

To avoid the inclusion of dogs with occult *D. immitis* infection, dogs showing positivity on any of the antigen tests were ruled out (even the ones with microfilaremia and negative PCR results for *D. immitis*). A combination test was used for the detection of antibodies against *Ehrlichia*, *Anaplasma*, and *Borrelia* infection (Personal-Lab, Italy or Siemens BEP 2000, Germany). As leishmaniasis is not endemic in our area dogs were not screened for this agent. We neither screened dogs for Leptospira infection, as all dogs were annually vaccinated, and strict anti-rodent control took place in both institutions.

Urine samples were taken by cystocentesis with a 22 or 23 G needle and a 5-10 ml syringe. A complete urinalysis was carried out, including microscopic evaluation of the urine sediment as well as the measurement of UPC and UAC. A urine culture examination was also carried out.

Urinary albumin, total protein and creatinine was measured the same way as previously described in Chapter 6.3.2.

Albuminuria was established at a UAC over 19 mg/g (Falus et al., 2022) and proteinuria at a UPC over 0.2 (Lees et al., 2005).

For those dogs where follow-up was feasible, we repeated the measures of UAC and UPC after one or two doses of moxidectin/imidacloprid treatment (Advocate spot-on, Elanco Animal Health Incorporated, Indiana, US [formerly Bayer Animal Health GmbH, Germany]; following the instructions of the manufacturer, 25 mg for dogs between 4-10 kg and 62.5 mg for dogs between 10-25 kg, with 4 weeks of interval between two treatments).

7.3.3 Statistical Analyses

The Shapiro-Wilk test was performed for all the measured variables, and data were expressed as mean and standard deviation for those with normal distribution or median and range/interquartile range (IQR) for the non-normally distributed data.

Our primary null hypothesis was that *D. repens* infected dogs have the same magnitude of proteinuria and albuminuria as non-infected dogs kept under the same circumstances. The Mann-Whitney U test was used to compare the distributions of the two groups in these respects. Statistical significance was set at $P < 0.025$.

The null hypothesis for the secondary outcome of our study was that the magnitude of protein or albuminuria will not change after eliminating *D. repens* infection. The Wilcoxon Signed Rank Test was performed to test this hypothesis. Statistical significance was set at $P < 0.05$. For the comparison of other variables between the infected and non-infected dogs, two-sample T-test was used for normally distributed data and the Mann-Whitney U test was used when the variables were not normally distributed. Statistical significance was set at $P < 0.05$. Statistical analysis was carried out using the "R 4.0.2" software.

7.4 Results

In total, 65 dogs were enrolled in the study. 22 dogs were excluded because of a positive *D. immitis* antigen test (n=11), elevated CRP or WBC (n=5), positive urine culture result or active urine sediment (n=5), and one dog was excluded because the results of the two Knott's tests were different. After exclusions there remained 26 *D. repens* infected and 17 control dogs. In the infected group, the median age was 3.8 (1.4) years (median (IQR)), and body weight was 10.8 ± 1.7 kg (mean ± SD). 17 female and 9 male dogs were present in this group. In the control group, the mean age was 2.6 ± 0.6 years (mean ± SD), and body weight was 11.4 ± 1.2 kg (mean ± SD). 8 female and 9 male dogs were present in this group. None of the dogs were spayed or castrated. Although both groups included young to middle-aged dogs, age significantly differed between the two groups ($p = 0.04$). Body weight did not differ between the two groups ($p=0.22$). The ratio of females was slightly higher in the infected group (65% vs. 47%).

In 32/43 (74%) dogs two Knott-test results were available. There was only one dog that had to be excluded because the first Knott's test was positive and the second negative. For the remaining 31 dogs the results of the two Knott's tests were identical.

There was a significant difference in the UAC median (IQR) values between the two groups: 12.5 (31.8) mg/g for the infected and 6.3 (9) mg/g for the control group (Figure 14).

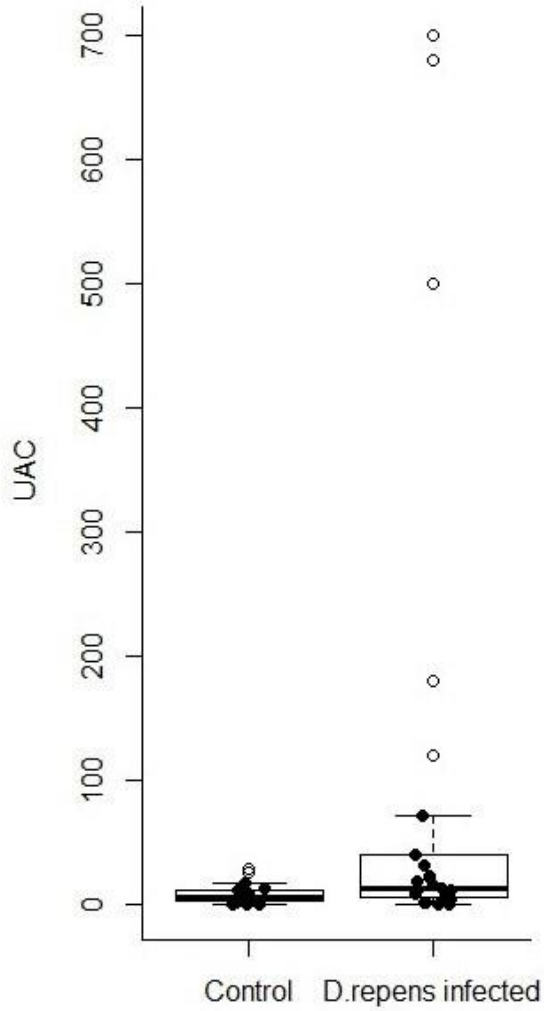

Figure 14. Urinary albumin to creatinine ratio (UAC; g/g) results of *D. repens* infected and control dogs.

Even though the UPC values were higher in the infected group (median 0.15 [IQR 0.31]) than in the control (median 0.13 [IQR 0.14]), this difference did not reach statistical significance (Figure 15).

Albuminuria (UAC >19 mg/g) was detected in 9/26 (35%) dogs in the infected group, while only 2/17 dogs (12%) had albuminuria in the control group. Proteinuria (UPC >0.2) was present in 11/26 (42%) of the infected dogs and 5/17 (29%) of the control dogs. Many dogs showed borderline proteinuria (UPC 0.2–0.5), while overt proteinuria (>0.5) was present in 6/26 (23%) of the infected dogs and 1/17 (6%) of the control dogs.

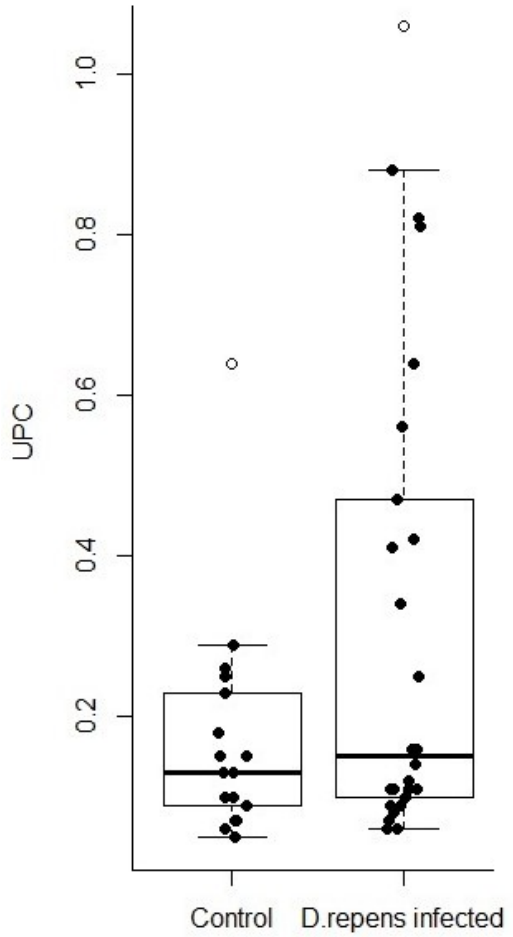

Figure 15. Urinary protein to creatinine ratio (UPC; g/g) results of *D. repens* infected and control dogs

Concerning other laboratory analytes, we found significantly higher eosinophil granulocyte count and platelet count in the infected group (see Table 8). 10/26 (39%) dogs in the infected group had elevated eosinophil cell count while only 2/17 (12%) had eosinophilia in the control group. Thrombocytosis was present in 11/26 (42%) dogs in the infected group and in 2/17 (12%) dogs in the control group. The UAC, UPC, platelet count and eosinophil cell count results of the two groups are shown in Table 8.

All other laboratory analytes were within the reference ranges. None of the infected dogs had to be excluded because of azotemia or signs of kidney failure. No difference was detected in the urea (p=0.40) or creatinine values (p=0.62), nor in the urine specific gravity results (p=0.62) between the two groups.

Table 8. The urinary albumin to creatinine ratio (UAC), urinary protein to creatinine ratio (UPC), thrombocyte count, and eosinophil cell count results of *D. repens* infected and control dogs.

	***D. repens* infected group n=26**	**Control group n=17**	**p**
UAC (mg/g)			
Median	12.5	6.3	0.02
Min; Max	0; 700	0; 28	
UPC (g/g)			
Median	0.15	0.13	0.65
Min; Max	0.06; 1.06	0.05; 0.64	
Platelet count (G/L)			
Mean ± SD			
Min; Max	439.4 ± 165.1	355.6 ± 84.9	0.003
	163; 874	218; 495	
Eosinophil cell count			
(G/L)	0.79 ± 0.36	0.45 ± 0.29	0.041
Mean ± SD	0.15; 1.5	0.1; 1.1	
Min; Max			

12 infected dogs were monitored after topical moxidectin treatment. Urine samples were collected one month after their first treatment and in 5 dogs one month after the second treatment as well. The UAC and UPC levels did not differ between any of the sampling time points. UAC was median 26.5; range, 11-700 mg/g at the first sampling time point, 41; 9-880 mg/g at the second (p=0.08) and 110; 3-1520 mg/g at the third time point (p=0.52). UPC was median 0.13; range, 0.08-1.06 at the first sampling time point, 0.33; 0.07-1.37 at the second (p=0.14) and 0.29; 0.08-3.85 at the third time point (p=0.54).

7.5 Discussion

Membranoproliferative glomerulonephritis is a known consequence of *D. immitis* infection. Albuminuria and proteinuria are the typical signs of glomerular dysfunction and are commonly associated with heartworm disease (Barsanti, 1977; Osborne et al., 1981; Buoro & Atwell, 1983; Grauer et al., 1987; Ludders et al., 1988; Grauer et al., 2002; Atkins et al., 2011).

Our study investigated the presence of albuminuria and proteinuria in *D. repens* infected dogs. We found a significantly higher amount of albumin present in the urine of the infected dogs.

Two previous studies found that microfilaremic dogs have more severe proteinuria than those without (Morchón, 2012; Hormaeche, 2014). In the study of Hormaeche et al., UPC values were 0.64 ± 0.86 for microfilaria negative and 0.93 ± 1.53 for positive dogs. In our study, all *D. repens* infected dogs were microfilaremic. Although there were more overt proteinuric dogs in the infected group than in control, the UPC values (median 0.15; min. 0.06; max. 1.06) were lower than those previously found in heartworm disease. There are many possible explanations for this. First, adult *D. immitis* worms stay in circulation and can have a much higher antigen stimulus with more immune complex formation than adult *D. repens* worms living in the subcutaneous tissue. Second, our dog population included mostly young adult dogs, and most probably, the duration of the infection also matters.

In the study of Grauer et al., twelve young beagle dogs were experimentally infected with *D. immitis* larvae. All dogs developed microalbuminuria within ten months (detected with the ELISA method). In the same study the overall number of proteinuric urine samples was 82%. In our study this number was 42%, while overt proteinuria was present in 7% in their study and in 23% in our study (Grauer et al., 2002). Albuminuria was present in 75% in the study of Grauer et al., while it was 35% in our study. In the study of Grauer et al., the group fed a diet with a 5:1 omega-6:omega-3 fatty acid ratio developed microalbuminuria later (9.7±1.0 months) than those with a 50:1 omega-6:omega-3 fatty acid ratio (7.5 ±1.9 months). Albuminuria increased with time in both groups and preceded proteinuria in all dogs (Grauer et al., 2002).

Cats infected with *D. immitis* infection were also found to be albuminuric. In the study of Atkins et al., 80 cats were experimentally infected with *D. immitis*. Albuminuria was detected by the ERD test 8 months post-infection. After exclusions 10/46 (22%) cats showed both albuminuria and significant proteinuria. These numbers are similar to our findings in *D. repens* infected dogs. Albuminuria ranged from 1 ("low positive") to 3 ("high positive") on the test (mean 1.5±0.85). Both microfilaraemic and amicrofilaraemic cats showed albuminuria, and there was a subtle association between worm burden and the degree of albuminuria.

UPC was elevated in all albuminuric cases (mean 0.57 ±0.91) (Atkins et al., 2011). This number is greater than what we found in *D. repens* infected dogs.

In our study, 9/26 (35%) dogs had albuminuria in the *D. repens* infected group and 2/17 (12%) dogs in the non-infected group. The two dogs from the non-infected group (26 and 28 mg/g) and three from the infected group had mild albuminuria (22, 23 and 32 mg/g). If we presume there is a borderline albuminuric range, these dogs had borderline albuminuria, which can mean early renal damage but can also be a transient and harmless laboratory change. The other 6/26 (23%) infected dogs had moderate to severe albuminuria (71, 120, 180, 500, 680 and 700 mg/g). This magnitude of albuminuria should already be taken seriously, and it is most probably a consequence of glomerular damage (with a lesser chance of tubular damage).

Interestingly, there were no differences in other investigated renal variables between the infected and non-infected animals. However, in the Ph.D. study of Olga Jacsó, *D. repens* infected dogs had higher urea levels than the non-infected dogs (Jacsó, 2014). This difference could be attributed to including older dogs in her research, while our dog population was relatively young. The longer duration of infection in older dogs could lead to more severe renal damage caused by the worms. Additionally, older dogs are more prone to other kidney-related problems, which could have contributed to the higher urea values. These potential causes highlight the need for further investigation.

As glomerular diseases are often present in non-azotaemic dogs with acceptable urine-concentrating ability, the presence of albuminuria increases the suspicion of glomerular damage caused by *D. repens* infection.

The higher eosinophil cell count in the infected group is likely explained by the parasite load. Eosinophilia is a frequent finding in heartworm-infected dogs with microfilaremia, too (Niwetpathomwat et al., 2007). Eosinophil cells are attracted by worms through type 2 cytokine IL-5 at the site of infection where they play a role in the elimination of the worms (Huang et al., 2016).

Thrombocytosis is described in *D. immitis* infection and other parasitic infections (e.g. *Spirocerca lupi*, *Angiostrongylus vasorum*) (Frank et al., 1997; Dvir et al., 2008; Silva et al., 2021). Thrombocytosis generally happens due to increased platelet production and release. In reactive thrombocytosis increased thrombopoietin or other inflammatory cytokines stimulate thrombopoiesis. Reactive thrombocytosis can be caused by neoplasia, chronic inflammatory diseases, immune-mediated diseases, trauma, or iron deficiency anemia (URL: https://eclinpath.com/hemostasis/disorders/platelet-numbers). In *D. repens* infection it could be explained by the inflammation and platelet activation induced by the migrating and circulating larvae or the adult worms within the skin.

To avoid any confounding factors, we chose young adult laboratory animals of the same breed kept and fed identically to compare two homologus groups that differ only in their *D. repens* infection status. Although most of the dogs in our study were young to middle-aged, there was a statistically significant difference between the age of the two groups (median 3.75 (IQR 1.38) years [infected] vs. 2.6 ± 0.6 years [control]). A previous study found that albuminuria can increase with age (Radecki et al., 2003), although the authors in this study only used a semi-quantitative test, and possible comorbidities – that are more frequent as dogs age – could have not been detected. In our reference interval study we could prove that there is no correlation between the UAC and age in healthy dogs (Falus et al., 2022).

We did not find a difference in the UAC and UPC levels after one or two moxidectin treatments. Many reasons may explain our findings. First, albuminuria and proteinuria could be related to the reaction provoked by the presence of adult worms. A single administration of topical moxidectin killed all microfilariae; however, six consecutive monthly treatments are needed to kill adult *D. repens* worms (Frangipane et al., 2016). This means that many adult worms were still alive after one or two moxidectin treatments. Second, dead worms could have provoked an inflammatory reaction leading to albuminuria or proteinuria. Third, more time could be needed for the suspected glomerular damages and albumin- or proteinuria to resolve, or if the damage is permanent, albumin- and proteinuria may never resolve. A limitation of this examination was that we did not collect blood samples (only urine samples) at the later sampling time points. Thus, any inflammatory reaction unrelated to the *D. repens* infection could have also increased the UAC or UPC results. The small number of dogs might have also distorted the results.

There are some limitations of the study. Urine culture examination was not carried out in 8 dogs, because of technical issues (3 in the infected and 5 in the control group). Nevertheless, all these dogs had inactive urine sediment without any lower urinary tract signs, thus including these dogs was justifiable in our opinion.

Another limitation was that 5 dogs were assigned to the control group with only one negative Knott's test (without having a prescreening Knott's test) and two negative *D. immitis* antigen tests. However, at the other dogs the results of the two Knott's tests done at different time points were identical in 97% of the cases. Thus, the possibility that we had included *D. repens* or *D. immitis* positive dogs in the control group was low. Nevertheless, even if it had happened, it would have only decreased the statistical difference and could not have been responsible for our findings.

In this study, we concluded that *D. repens* infection causes moderate-to-severe albuminuria in around one-quarter of the infected dogs and that proteinuria is less frequent and less severe in *D. repens* infection than in *D. immitis*.

Additional studies are needed to assess the significance of albuminuria in *D. repens* infected dogs. Histopathologic evaluation would be necessary to evaluate the presence and type of glomerular damage caused by *D. repens*.

8. New scientific results

1. Our study was the first to establish a reference interval of dogs' urinary albumin-to-creatinine ratio (UAC) based on the analysis of UAC in 124 healthy dogs, providing a solid foundation for future research and clinical practice:

   The reference interval for canine UAC is 0 – 19 mg/g. The human reference interval is somewhat higher: 0 – 30 mg/g, but very similar to the upper limit 90% confidence interval of canine UAC 13–28 mg/g, found in this study.

2. Breed, age, sex, body weight, or collection method does not seem to influence canine UAC values:

   The results of Sighthounds (n=30) and Beagle dogs (n=23) were also analyzed as subgroups. Sighthounds are known to have unique reference intervals, and Greyhounds were previously shown to excrete more albumin than other breeds. Breed-specific reference intervals for Beagle dogs could be helpful because of their frequent use as laboratory animals. We found no significant differences in the UAC values between Beagles, Sighthounds, and the rest of the dogs. In contrast to previous studies, the UAC did not show a significant association with the age of the animals. For detecting albuminuria, clinicians can decide between free-catch and cystocentesis methods based on which method is technically more accessible, safer, and more comfortable for the animal.

3. *D. repens* infected dogs excrete a higher amount of albumin in the urine than non-infected dogs:

   Albuminuria (UAC >19 mg/g) was detected in 35% of the dogs in the *D. repens* infected group, while only 12% had albuminuria in the control group. Although there were more overt proteinuric dogs (UPC >0.5) in the infected group (23%) than in control (6%), the UPC values did not significantly differ between the infected and control groups. Albuminuria raises the suspicion of early glomerular damage caused by *D. repens* infection, similarly to *D. immitis*.

4. *D. repens* infection causes increased eosinophil granulocyte count and platelet count, while it does not seem to cause azotemia or decreased urine specific gravity:

   Eosinophilia was present in 39%, and thrombocytosis in 42% of the *D. repens* infected dogs (vs. 12% and 12% in the control group). Our findings of physiologic serum creatinine and urea values and urine specific gravity do not exclude glomerular damage, as glomerular diseases are often present in non-azotaemic dogs with acceptable urine-concentrating ability.

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10. Scientific publications

10.1 Publications in peer-reviewed journals related to the thesis

Falus F.A., Szabó K. É., Becker Zs., Müller L., Fok É., Balogh N., Manczur F. 2023. Albuminuria and proteinuria in dogs infected with *Dirofilaria repens*: A cross-sectional study. Journal of Veterinary Internal Medicine, 37(3), 992-997.

Falus F.A., Vizi Zs. Szabó K.É., Müller L., Reiczigel J., Balogh N., Manczur F. 2022. Establishment of a reference interval for urinary albumin-to-creatinine ratio in dogs. Veterinary Clinical Pathology, 51(4), 585–590.

Falus F. A., Manczur F. 2018. Kutyák és macskák proteinuriája II. rész: Kutyák és macskák proteinuriájának gyógykezelése: Irodalmi összefoglaló. Proteinuria in dogs and cats Part 2. Treatment of proteinuria in dogs and cats. Literature review. Hungarian Veterinary Journal, 140 (3), 135-149.

Falus F.A., Székely D. Manczur F. 2017. Kutyák és macskák proteinuriája: Irodalmi összefoglaló I. rész. Proteinuria in dogs and cats Part 1. Literature review. Hungarian Veterinary Journal, 139(2) 89-100.

10.2 Scientific posters, presentations

Falus F.A., Vizi Zs., Török B., Manczur F. 2020. Evaluation of the diagnostic value of urinary albumin to protein ratio in proteinuric dogs. Research Communications of the 29th ECVIM-CA Congress, Journal of Veterinary Internal Medicine 34 (1).

Manczur F., **Falus F. A.**, Kubik N., Müller L., Vizi Zs.; Sterczer Á., Rabnecz Gy. 2018. Microalbuminuria in dogs infected with *Dirofilaria repens*. Research Communications of the 27th ECVIM-CA Congress, Journal of Veterinary Internal Medicine 32 (1) 531.

Manczur F., **Falus F.A.**, Vizi Zs. 2017. Microalbuminuria in healthy dogs. Research Communications of the 26th ECVIM-CA Congress, Journal of Veterinary Internal Medicine 31 (1) 253.

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12. Supplementary material

Table S1. Data of dogs in the urinary albumin-to-creatinine (UAC) reference interval study.

Name	Breed	Bodyweight	Age (years)	UAC (mg/g)
Fülemüle	Hungarian greyhound	22	5,1	2
Felleg	Hungarian greyhound	26,8	5,8	6
Fodorka	Hungarian greyhound	26,5	7,1	0
Írisz	Hungarian greyhound	26,5	1,9	2
Ibolya	Hungarian greyhound	27,2	1,9	0
Vakvákta kecses	Hungarian greyhound	26,5	3,5	0
Csipke	Hungarian greyhound	25	6,9	0
Rozsda	Hungarian greyhound	26	4,1	7
Egyetlen Apró Angyal	Hungarian greyhound	26,5	2,8	3
Szusza	Hungarian greyhound	25,4	2,8	1
Álmi	Hungarian greyhound	23,4	2,8	24
Csinos	Hungarian greyhound	25,4	7,4	4
Nimród	Hungarian greyhound	28,5	6,2	2
Iglice	Hungarian greyhound	25,2	2,2	7
Kacaj	Hungarian greyhound	24	2,7	6
Ejha	Hungarian greyhound	21	5,1	2
Emlék	Hungarian greyhound	23	5,1	3
Bori	Hungarian greyhound	31,5	4,5	1
Fürge	Hungarian greyhound	26	7,1	10
Góliát	Hungarian greyhound	34,8	7,9	20
Gólya	Hungarian greyhound	30,6	6,7	4
Burián	Borzoi	-	4,7	1
Zsivány	Hungarian greyhound	29	6,7	4
Jasma	Borzoi	-	2,7	0
Csipke	Hungarian greyhound	-	2,7	1
Jura	Hungarian greyhound	28,5	7,9	39
Béla	Hungarian greyhound	26,8	3,9	3
William	Galgo Español	8	3,7	9
Almasnaja	Hungarian greyhound	23,7	5,1	3
Suri	Whippet	10,5	1,4	4
Ayana	Deutsch Drahthaar	20,6	3,5	0
Berci	mixed breed	30	6,6	0
Madzag	Dachshund	5,6	3,6	48
Lucky	Briquet Griffon Vendéen	24	2,0	2
Bundika	mixed breed	5	3,6	0
Lily	mixed breed	24,4	2,0	0
Tüsi	mixed breed	9,4	5,4	6
Maci	mixed breed	21,5	4,5	0

Saffi	mixed breed	15	1,6	6
Bernie	mixed breed	18,5	2,8	0
Panni	mixed breed	23,5	1,0	2
Morci	mixed breed	15	2,6	0
Ellie	mixed breed	26,1	3,9	0
Millie	Collie	17,8	3,0	1
Viki	mixed breed	16,6	1,7	0
Tanuki	Akita	29,6	1,5	1
Darwin	Akita	-	4,0	2
Happy	mixed breed	11	5,4	0
Böbe	mixed breed	18,8	5,7	2
Artisz	Weimaraner	32,5	2,0	1
Kira	mixed breed	20,8	4,4	2
Stefi	Dachshund	7	3,8	11
Műzli	Miniature Pinscher	9	6,7	6
Charlie	mixed breed	11	5,0	0
Leon	mixed breed	6,6	3,0	2
Sam	Collie	25	4,0	4
Hope	Border Collie	18	2,0	8
Dany	Dogo Argentino	38	4,0	21
Rozi	Deutsch Drahthaar	23,5	9,1	14
Sába	German sheperd	26	2,3	2
Johnny	Bullterrier	26,4	4,1	11
Balu	Labrador retriever	38	4,7	4
Liza	American staffordshire terrier	35	5,4	6
Rozi	Golden retriever	26,5	5,3	9
Hádész	Dogo Argentino	39,2	3,1	7
Rini	Bernese mountain dog	37,4	7,0	1
Zira	Doberman	32	2,8	4
Indy	Labrador retriever	28,5	2,3	3
Hektor	French bulldog	15,8	5,2	5
Rico	mixed breed	17,2	4,1	4
Betty	Schnauzer	9,8	3,6	7
Cappy	Border Collie	19,4	8,6	2
Qitty	Malinois	21	2,2	5
Jenny	Deutsch Drahthaar	32,5	4,8	6
Pixel	Australian kelpie	16	2,2	4
Archibald	mixed breed	10,2	4,5	8
Lujzi	mixed breed	11	6,9	7
Maci	mixed breed	15,4	4,5	12
Níla	Australian kelpie	13,6	2,5	5
Mozart	Golden retriever	31,8	7,2	7
Jamie	Jack russel terrier	6	6,7	14

Rico	Parson russel terrier	8,1	5,6	13
Imbi	mixed breed	20	6,9	3
Sprite	Golden retriever	26,5	1,1	3
Gino	mixed breed	8,6	4,3	5
Suri	mixed breed	10	1,5	4
Zazi	Hungarian vizsla	25	1,3	1
Delon	Border Collie	20,9	5,9	11
Szilvia	mixed breed	24	3,2	10
Luna	Deutsch Drahthaar	23,5	7,0	13
Dorka	Hungarian vizsla	26,3	6,4	1
Pascal	Golden retriever	43	4,1	6
Fiona	German sheperd	28	5,4	4
Mable	Border Collie		7,5	0
Audry	Bedlington terrier	9	6,4	0
Bundika	mixed breed	5,5	10,7	0
Sunny	Cavalier king Charles spaniel	5,5	8,4	1
Winny	Miniature Pinscher	5,5	2,1	0
Bodza	mixed breed	8,2	3,4	0
Monroe	Dutsch Shepherd	29	4,6	0
Málna	Malinois	-	7,2	0
X6125	Beagle	14	2,0	5
9824	Beagle	11,5	3,0	13
2352	Beagle	10,7	3,0	6,3
2105862	Beagle	9,4	2,0	3
H5d7473	Beagle	-	2,0	2
H5d7491	Beagle	-	2,0	3
H6a4889	Beagle	-	5,0	21
7400	Beagle	13	2,5	12
7582	Beagle	11,4	2,0	0
7326	Beagle	10,8	3,0	28
5439	Beagle	11,7	3,5	7
7534	Beagle	11,8	2,0	8
7462	Beagle	11,3	2,5	3
2449430	Beagle	9,5	2,5	1
2444225	Beagle	9,5	2,5	8
2446665	Beagle	10	3,0	0
8624	Beagle	10,6	1,9	9
8641	Beagle	8,7	1,9	1
8671	Beagle	8,6	1,8	0
8638	Beagle	9,9	2,0	1
8648	Beagle	10,6	1,9	1
8639	Beagle	10,1	1,9	1
8637	Beagle	8	1,9	1