Chronic kidney disease in the dog

Pathophysiological mechanisms and diagnostic aspects

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Chronic kidney disease in the dog – pathophysiological mechanisms and diagnostic aspects

Abstract

Chronic kidney disease (CKD) is a contributor to morbidity and mortality in dogs. The general aim of this thesis was to increase knowledge regarding pathophysiologic mechanisms and early diagnosis of canine CKD by identifying dogs with increased risk of disease, and by exploring the value of various biomarkers in blood and urine.

Dogs are comparably commonly diagnosed with kidney-related disease (KD), but neither incidence nor mortality rates of KD have previously been reported. In paper I, incidence and mortality rates of KD were calculated in a population of >600,000 dogs. The total incidence rate of KD in this population was 15.8 cases per 10,000 dog years at risk (DYAR, representing one dog insured for one year). The mortality rate of KD was 9.7 deaths per 10,000 DYAR. The breeds with the highest incidence rates in this study were the Bernese mountain dog, miniature schnauzer and boxer. The Swedish elkhound, Siberian husky and Finnish spitz were the breeds with the lowest rates.

Increased concentrations of two cardiovascular biomarkers, B-type natriuretic peptide (NT-proBNP) and cardiac troponin I (cTnI), have been reported in dogs with decreased renal function. The aim of paper II was to investigate if NT-proBNP and cTnI accumulate in the circulation of dogs with CKD, as GFR declines. The results did not support passive accumulation, and the conclusion was that these markers identify increased blood volume and damage to cardiac cells, respectively, in dogs with CKD.

Symmetric dimethyl arginine (SDMA) and cystatin C are two potential biomarkers of decreased GFR in the dog. In paper III, the aim was to investigate overall diagnostic value of SDMA and cystatin C as markers of decreased GFR, compared to the current marker, creatinine. The overall value of SDMA was equivalent to that of creatinine, but cystatin C performed less well as a marker of decreased renal function in this study.

In human medicine, a specific urinary peptide pattern that detects CKD has been developed. Changes in the canine urinary peptide pattern may represent a completely new opportunity for early diagnosis of canine CKD as well. In paper IV, a CE-MS-based urinary peptidome model, 133P, was constructed and shown to be able to discriminate healthy dogs from dogs with CKD in a separate cohort. This model, although in need of further investigation and validation, represents an exciting new diagnostic modality in that it may prove to be able to detect chronic progressive CKD in a single urine sample.

Keywords: CKD, renal, canine, biomarker, GFR, cardiovascular-renal disorder, nephrology

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Kronisk njursjukdom hos hund – patofysiologiska mekanismer och diagnostiska aspekter

Sammanfattning

Kronisk njursjukdom (CKD) hos hund är en allvarlig sjukdom som kan ha många olika underliggande orsaker. De övergripande målen med denna avhandling var att öka kunskapen kring patofysiologi och tidig diagnostik av CKD hos hund. I studie I sammanställdes data från > 600 000 hundar registrerade hos Agria Djurförsäkring under 12 års tid. Förekomst av njursjukdom beräknades till ca 16 nya fall per 10 000 hundår (ett mått som innebär en hund försäkrad under ett år). Dödlighet till följd av njursjukdom uppgick till ca 10 dödsfall per 10 000 hundår. Högst förekomst av njurrelaterad sjukdom sågs hos berner sennen, dvärgschnauzer och boxer. Lägst förekomst sågs hos jämthund, siberian husky och finsk spets.

B-type natriuretic peptide (NT-proBNP) och hjärtspecifikt troponin I (cTnI) är två sjukdomsmarkörer som kan mätas i blodet och används för att upptäcka hjärt- och kärlpåverkan hos hund. Tidigare studier har visat att höga koncentrationer av dessa markörer kan ses hos hundar med njursjukdom. Anledningen till detta är inte känd, men en passiv ackumulering av NT-proBNP och cTnI i blodet har misstänkts, vilket undersöktes i studie II. Resultaten indikerade att en passiv ackumulering av dessa markörer inte föreligger hos hund när njurfunktionen minskar. Detta innebär att NT-proBNP och cTnI påvisar hjärt- och kärlpåverkan även hos hundar med CKD.

Symmetric dimethyl arginine (SDMA) och cystatin C utgör två markörer för detektion av nedsatt njurfunktion (GFR). I studie III undersöktes det diagnostiska värdet av dessa markörer jämfört med den för närvarande vanligaste markören för njurfunktion, kreatinin. Det övergripande diagnostiska värdet av SDMA var likvärdigt med värdet av kreatinin. Värdet av cystatin C var lägre än det av de båda andra markörerna. För vissa hundar finns dock anledning att analysera SDMA eller cystatin C i tillägg till kreatinin.

I studie IV utvärderades en helt ny metod (kapillär elektrofores och masspektometri, CE-MS) för diagnosticering av njursjukdom. Med hjälp av denna metod undersöks urinens hela innehåll av peptidfragment. Skillnader i peptidmönster mellan sjuka och friska individer undersöktes och en statistisk modell, 133P, för diagnostik av CKD konstruerades. Validering av 133P i en separat grupp av hundar med och utan CKD visade att modellen kunde särskilja dessa grupper. Urinanalys med hjälp av CE-MS behöver valideras ytterligare, men utgör ett potentiellt nytt alternativ för tidig diagnostik av CKD hos hund i framtiden.

Nyckelord: CKD, nefrologi, kardiorenalt syndrom, njursvikt, GFR, biomarkör

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To my parents, Birgitta & Göte

Medicine is a science of uncertainty and an art of probability

William Osler

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Pelander, L.*, Ljungvall, I., Egenvall, A., Syme, H., Elliott, J., Häggström, J. (2015). Incidence of and mortality from kidney disease in over 600,000 insured Swedish dogs. *Veterinary Record*, 176(25), p. 656.

II Pelander, L.*, Häggström, J., Ley, C.J., Ljungvall, I. (2017). Cardiac troponin I and amino-terminal pro b-type natriuretic peptide in dogs with stable chronic kidney disease. *Journal of veterinary internal medicine*, 31(3), p. 805-813.

III Pelander, L.*, Häggström, Larsson, A., Syme, H., Elliott, J., Heiene, R., Ljungvall, I. Diagnostic evaluation of cystatin C, symmetric dimethylarginine and creatinine for detection of decreased GFR in 97 dogs. Submitted manuscript.

IV Pelander, L.*, Brunchault, V., Buffin-Meyer, B., Klein, J., Breuil, B., Zürbig, P., Magalhães P., Mullen, W., Elliott, J., Syme, H., Schanstra, J.P., Häggström, J., Ljungvall,I. Urinary peptidome analyses for diagnosis of chronic kidney disease in dogs. Submitted manuscript.

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The contribution of Lena Pelander to the papers included in this thesis was as follows:

I Participated in planning of the study and result interpretation. Wrote the manuscript with input from co-authors.

II Participated in planning of the study. Recruited and managed cases and control dogs. Conducted the statistical analyses and wrote the manuscript with input from co-authors.

III Took major part in planning of the study. Recruited and managed cases and control dogs. Conducted the statistical analyses and wrote the manuscript with input from co-authors.

IV Took major part in planning of the study. Recruited and managed cases and control dogs. Participated in result interpretation. Wrote the manuscript with input from co-authors.

Related work not included in the thesis

- Mattei, C., Pelander, L., Hansson, K., Uhlhorn, M., Häggström, J., Ljungvall, I., Ley, C. J. Comparison of ultrasonographic abnormalities with GFR in canine stable CKD. Abstract presented (C.M.) at ECVDI-congress, June 2017.

Abbreviations

^{99}Tc-DTPA	Technetium-99m-dietylene-triaminepentaacetic acid
AKI	Acute kidney injury
AUC	Area under the (receiver operator) curve
BW	Body weight
CE-MS	Capillary electrophoresis and mass spectrometry
CI	Confidence interval
CKD	Chronic kidney disease
cTnI	Cardiac-specific troponin I
CvRD	Cardiovascular-renal axis disorders
DYAR	Dog years at risk
ECM	Extra-cellular matrix
eGFR	Estimated glomerular filtration rate
GFR	Glomerular filtration rate
IR	Incidence rate
IRIS	International renal interest society
KD	Kidney-related disease
LMW	Low molecular weight
LR	Likelihood ratio
LR-	Negative likelihood ratio
LR+	Positive likelihood ratio
LR_i	Interval likelihood ratio
MBD	Mineral and bone disease
mGFR	Measured glomerular filtration rate
MMP	Matrix metalloproteinase
MR	Mortality rate
NT-proBNP	N-terminal pro B-type natriuretic peptide
PCV	Packed cell volume (haematocrit)
PU/PD	Polyuria and polydipsia

PVf/kg	Plasma volume factor indexed to body weight
RAAS	Renin angiotensin aldosterone system
RCV	Reference change value
ROC	Receiver operating characteristic
SBP	Systolic blood pressure
SDMA	Symmetric dimethyl arginine
SVM	Support vector machines
UPC	Urine protein-to-creatinine ratio
USG	Urine specific gravity

1 General background

Chronic kidney disease (CKD) is an important contributor to morbidity and mortality in dogs. The definition of CKD has changed over time and is not clearly established in veterinary medicine. In some companion animal medicine textbooks, CKD is defined as *presence of functional or structural damage to one or both kidneys with a duration of more than three months* (Polzin, 2017), which is similar to the definition of human CKD (Levey, 2012). Structural damage refers to abnormalities of macroscopic or microscopic anatomy. The term "renal function" most commonly refers to the glomerular filtration rate, GFR, which is considered the best indicator of global renal function. However, the kidneys continuously perform other multiple, life essential tasks in order to maintain whole-body homeostasis. Tubular actions (reabsorption, secretion, and electrolyte and acid-base homeostasis), glomerular filtration barrier integrity and different endocrine functions are examples of "renal function". Serious compromise of one or more of these functions may be present without a detectable decrease in GFR (Klosterman *et al.*, 2011; McNamara *et al.*, 1989).

As renal functional mass decreases, multiple systemic negative consequences develop. Some of those may also further compromise the kidney. Detection of these systemic consequences is important both diagnostically and for optimal treatment. In both human and veterinary medicine, CKD is regarded, and often described as, a progressive, incurable disease (Jepson & Syme, 2017). This is however not true for all dogs that fit the currently used CKD definition.

There are multiple possible underlying aetiologies of canine CKD. In many cases, the initiating cause is not evident, and may no longer be present, at the time of diagnosis. Clinical signs of CKD are not pathognomonic and, at least for some diseases of the kidney, often do not emerge until comparably late in the course of disease, because of the large reserve capacity of these organs.

In order for dogs to be investigated for CKD, they must be presented to a veterinarian by their owner. Awareness among owners and veterinarians of the general incidence of CKD and any increased risk of the disease in certain breeds

probably increases the opportunity for early diagnosis. Reported prevalence of CKD in canine studies varies, depending on the population studied (Sosnar *et al.*, 2003; Lund *et al.*, 1999). A reasonable estimate of overall prevalence of CKD in the canine population has been suggested to be 0.5-1.5% but there are no studies that confirm this statement (Brown, 2007). Because of the insidious character of the disease and the commonly delayed diagnosis, estimates of prevalence may be falsely low. Breed predisposition to specific diseases of the kidney are known for a few breeds and suspected in others, but differences in incidence or mortality rates of kidney disease between breeds have previously not been investigated.

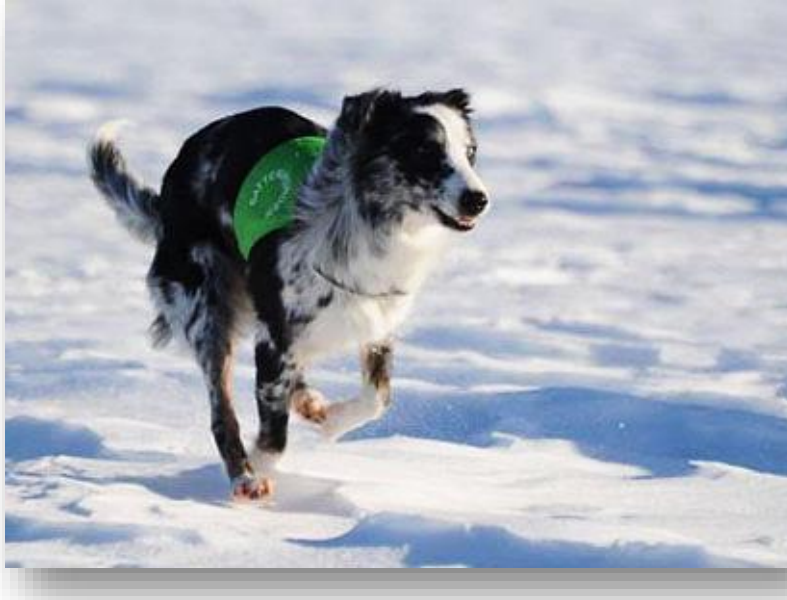
Current methods for diagnosis of canine CKD are insensitive, especially for some kidney disease manifestations (such as chronic tubulointerstitial disease). Many of the diagnostic biomarkers that are used, are also not specific for renal dysfunction and damage. Pre-renal and post-renal influences on both GFR and proteinuria are examples of states that complicate biomarker use (Tabaru *et al.*, 1993; Harris & Gill, 1981). Knowledge of renal physiology and pathophysiology is required for interpretation of test results.

Considerable effort is invested in potential new diagnostic methods for a number of reasons. A method for early diagnosis of CKD would be of value in the work-up of dogs that are presented with clinical signs such as chronic unexplained polyuria and polydipsia (PU/PD), or recurrent infection of the urinary tract. In both scenarios, subclinical CKD is a plausible differential diagnosis that often is difficult to diagnose (or rule out). Also, in breeds of dog that are predisposed to CKD of different aetiologies (for which a genetic test is not available, such as renal dysplasia), there might be uncertainty regarding kidney disease status in a certain young individual intended for breeding. Under these circumstances an early, robust method of diagnosis would be highly valuable in order to know which individuals not to breed.

Clinical management of CKD is aimed to resolve (if possible) any identified underlying pathology, provide adequate nutrition, slow progression of disease and prevent and treat complications associated with decreased renal function (Polzin, 2013). Owners of dogs with CKD are often willing to initiate chronic treatment in an attempt to prolong life, as long as quality of life is ensured. An early diagnosis of progressive disease probably increases the opportunity of more effective clinical management (Schievink *et al.*, 2016; Polzin, 2013), which may then focus on prolonging *time until development* of clinical signs. If a positive effect (such as slowing the rate of progression) of specific treatment is proven for dogs at the earliest stages of CKD, the value of early diagnosis would further increase. In order to evaluate that, however, dogs need to be diagnosed early in the disease process. A balance between “need and actions” is

important in this respect, because a diagnosis is only relevant if the disease is anticipated to generate morbidity in the future.

The ultimate, not yet accomplished, goal of CKD treatment in dogs, as well as in people, is to completely halt or even reverse disease progression (Tampe & Zeisberg, 2014). Increased knowledge regarding biological processes underlying CKD progression and the associated renal fibrosis is needed in order to reach this treatment goal in the future. Consequently, a continued effort to find new, robust methods of early diagnosis of CKD in dogs is desirable.



Joy, Australian shepherd, 2007-2016. One of the dogs that contributed with valuable data to this thesis. (Courtesy of Steff Krusengren).

2 Kidney anatomy and physiology

Anatomically, the kidneys consist of an outer region, the cortex covered by a capsule, and an inner region, the medulla, which embraces a centrally situated pelvis (Fig 1). The kidneys are responsible for many different aspects of homeostasis and may be divided into three functionally different compartments; the glomerular, the tubular and the interstitial compartments. Some authors also consider the renal vasculature a fourth compartment. The principal function of the kidney is to clear unwanted substances from the body, either by glomerular filtration, or by active secretion into the urine by the tubular system, or both. Examples of unwanted substances are end-products of metabolism such as inorganic phosphate and potassium and exogenous substances such as drugs and toxins. The kidneys are responsible for fluid-, acid/base- and electrolyte homeostasis as well as blood pressure regulation. Also, the kidneys perform multiple endocrine functions: Interstitial fibroblasts produce erythropoietin (EPO), a hormone needed for production of red blood cells (Kurtz *et al.*, 1989). The juxtaglomerular cells within the kidney produces the hormone renin in response to reduced circulating volume (Kurtz, 2011). Renin initiates a cascade called the renin-angiotensin-aldosterone system (RAAS), aimed at sodium and water retention. Also, the inactive metabolite 1, 25 dihydroxycholecalciferol is transformed into calcitriol, "activated" vitamin D, by 1α-hydroxylase, an enzyme of which the kidney is the most abundant source.

Each canine kidney contains several hundred thousand functional units called nephrons. Every nephron consists of a renal corpuscle (glomerulus, mesangium and Bowman´s capsule, Fig 2) and a tubular system, through which urine is transported towards the renal pelvis. The tissue between nephrons and vessels, the interstitium, acts as a support structure for cells. It consists of a highly charged extra-cellular matrix (ECM), which is most prominent in the renal medulla. Proteases (for example matrix metalloproteinases, MMPs) and their inhibitors maintain the delicate equilibrium between ECM synthesis and

degradation (Aresu *et al.*, 2011). It is now recognised that the ECM, in addition to its supportive function, also is highly active in cell signalling (Seikrit *et al.*, 2013; Rozario & DeSimone, 2010).

Through the renal arteries, the kidneys receive a sizable part (approximately 20%) of cardiac output, especially considering the small size (about 1% of body mass) of these organs. After flowing through interlobar, arcuate and interlobular arteries, blood enters the glomerular tuft, a web of fenestrated capillaries, where filtration takes place. Mesangial cells, which have contractile properties and are thought to contribute to the glomerular filtration function, provide structure for the glomerular capillary loops (Schlondorff, 1987). Glomerular plasma flow and intraglomerular pressure are fine-tuned by hemodynamic actions of the afferent and efferent arterioles at both ends of the glomerular tuft (Brown *et al.*, 1990). Blood is filtrated through three layers; the fenestrated endothelium, the glomerular basement membrane and the epithelium, through the slit diaphragm of podocytes (Deen *et al.*, 2001).

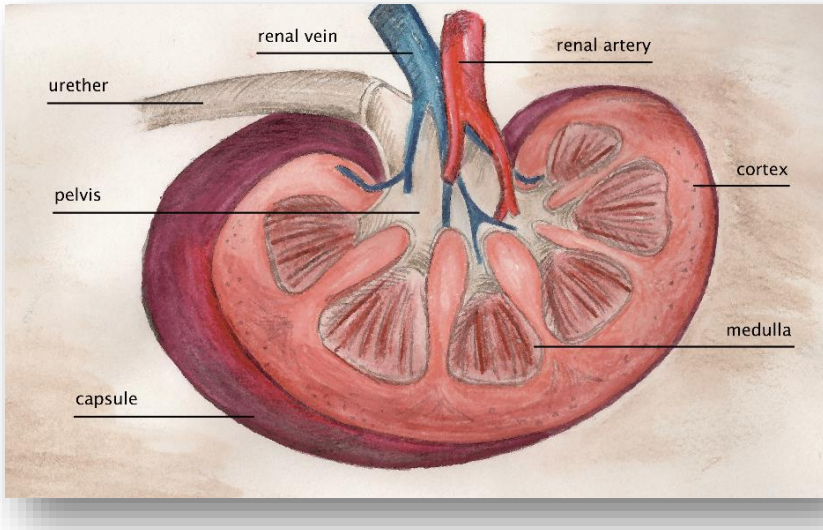


Fig 1. Macroscopic anatomy of the kidney. (Courtesy of Mike Dalbert *www.treeline.ch).*

These layers together provide a size- and charge-selective barrier (Brenner *et al.*, 1977). Hormonal, neural and vasoactive substances influence renal blood flow and glomerular filtration. Low molecular weight (LMW) molecules with radii < 20-25 Å (or less than ≈ 25 kDa) are relatively freely filtered, and those with radii >50-55 Å (or weights of more than ≈ 70 kDa) are to a great extent excluded from filtration (D'Amico & Bazzi, 2003; Oliver *et al.*, 1992; Maack *et al.*, 1979).

Apart from size and charge, the sieving coefficient (glomerular permeability) of different substances, especially medium-sized ones, also depends on the flexibility and shape of circulating molecules (Lindstrom *et al.*, 1997; Maack *et al.*, 1979).

The fraction of plasma that is filtrated depends on glomerular plasma flow, intraglomerular hydrostatic pressure, and ultrafiltration coefficient Kf (where Kf is the product of filtration barrier permeability and surface area) (Deen *et al.*, 1972). The filtrated plasma (ultrafiltrate, or primary urine) enters Bowman´s capsule, which constitutes the first part of the renal tubular system. The rate of filtration by the glomeruli, or the GFR, is defined as the amount of ultrafiltrate that forms in the nephrons per unit of time. The level of GFR in an individual person or animal with healthy kidneys is set by the metabolic rate (Singer, 2001).

Bowman´s capsule opens up into the proximal tubules, where 66-75% of the ultrafiltrate is reabsorbed, including approximately 60% of filtered sodium, potassium and chloride, 70% of filtered calcium, and 80% of filtered phosphate and bicarbonate (Boron, 2006; Duarte & Watson, 1967; Malnic *et al.*, 1964). Glucose and amino acids, such as cystine, ornithine, lysine and arginine are almost completely reabsorbed in the proximal tubular segment (Silbernagl, 1988). Small filtered proteins such as albumin (most of which is retained from filtration, but filtered in small amounts in healthy individuals), are reabsorbed into proximal tubular cells by megalin-mediated pinocytosis (Vinge *et al.*, 2010; Lazzara & Deen, 2007).

In the remaining parts of the tubular system, further concentration of urine and necessary adjustment of acid/base-, mineral- and electrolyte-status occurs. The filtrate then enters the renal pelvis and the urethra for transport to the urinary bladder as urine. Blood that is not filtered in the glomerulus and instead enters the efferent arteriole flows through another, peritubular, capillary system. These capillaries provide oxygen and nutrition to the renal parenchyma, including the tubular system (Beeuwkes & Bonventre, 1975).

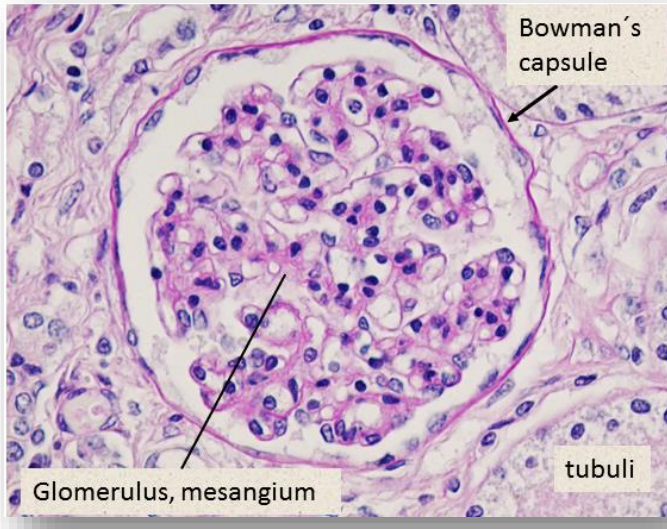


Fig 2. Normal glomerulus. Light microscopy image of a canine renal corpuscle (glomerulus) and the surrounding tubuli. Periodic acid-Schiff stain. (Courtesy of Fredrik Södersten).

3 CKD in dogs

Disease affecting the kidneys has traditionally been grouped in two main forms according to the clinical course; acute kidney injury (AKI) and CKD. Chronic kidney disease is often regarded a result of repeated, smaller insults to the kidney (Nenov *et al.*, 2000). However, one major injury may also lead to development of CKD (Venkatachalam *et al.*, 2010). Consequently, AKI may result in CKD, but is also a complication thereof, in that an individual with CKD may be predisposed to further injury, "acute-on-chronic disease" (Venkatachalam *et al.*, 2010). Recently, an even closer interrelation between AKI and CKD has been proposed in both human and veterinary medicine (Cowgill *et al.*, 2016; Chawla & Kimmel, 2012). It has been suggested that mechanisms of pathogenesis may be shared between the two. If this is true, CKD may be thought upon as a slowly (or of variable pace) progressing acute, or ongoing, kidney injury (Cowgill *et al.*, 2016).

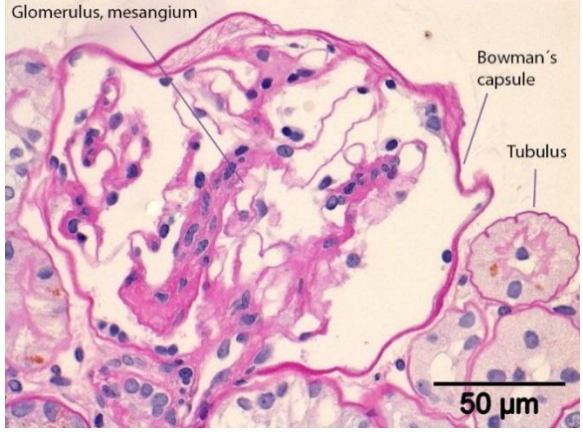


Fig 3. Diseased glomerulus. Light microscopy of a renal biopsy sample from a miniature poodle included in paper III. Morphological lesion (identified by use of electron microscopy): Focal segmental glomerulosclerosis. (Courtesy of the European Veterinary Renal Pathology Service). Periodic acid- Schiff stain.

Chronic kidney disease may be broadly divided into glomerular or tubulointerstitial disease. In glomerular disease, dysfunction of the filtration barrier is present (Fig 3). Thus, the hallmark of glomerular disease is renal protein loss (renal proteinuria). Glomerular damage may be categorised as immune-complex glomerulonephritis (ICGN) or non-ICGN (amyloidosis, focal segmental glomerulosclerosis) (Cianciolo *et al.*, 2016). Multiple genetic abnormalities that result in glomerular dysfunction have also been described (Littman *et al.*, 2013; Nowend *et al.*, 2012; Davidson *et al.*, 2007).

Tubulointerstitial disease refers to a disease process present in any area of the kidney apart from the glomerulus and pelvis, and examples that occur in dogs are renal dysplasia and Fanconi syndrome (Hoppe & Karlstam, 2000; Bovee *et al.*, 1978). Chronic tubulointerstitial fibrosis is also the final pathway of CKD irrespective of underlying pathology, and the degree of tubulointerstitial damage is the morphological feature that is most closely associated with GFR (Nath, 1992; Schainuck *et al.*, 1970).

3.1 CKD aetiologies

Many different underlying mechanisms of renal damage may lead to CKD. These include inflammatory, immune mediated, infectious, vascular, metabolic or neoplastic disease, toxicity, trauma, and genetic predisposition. The exact aetiology is often not known in dogs that are given a clinical diagnosis of kidney disease.

3.2 Disease development

During the clinical course of progressive CKD when nephrons are continuously lost, remaining nephrons undergo hypertrophy. Single nephron GFR increases (glomerular hyperfiltration) because of afferent (and to a lesser extent, efferent) arteriolar relaxation (Brown *et al.*, 1990; Deen *et al.*, 1974). These adaptive responses may initially be considered beneficial in maintaining global GFR, but over time, glomerular hypertrophy and hyperfiltration are thought to contribute to glomerulosclerosis and further nephron loss (Finco *et al.*, 1999; Brown *et al.*, 1990; Brenner *et al.*, 1982). At what level of decrease in nephron mass the inherent, self-perpetuating progression starts is not known. Approximately 31/32 removal of renal mass in dogs results in moderate azotaemia (≈177-253 µmol/L) after allowing time for compensatory hypertrophy and hyperfiltration (Finco *et al.*, 1999; Finco *et al.*, 1994; White *et al.*, 1991). This degree of azotaemia is common in dogs diagnosed clinically with CKD. Consequently, it has been

suggested that self-perpetuating disease may be present in many dogs at diagnosis (Finco *et al.*, 1999).

Variables associated with progression

Several clinicopathological variables have been associated with progression in dogs and people (Fig 4). Proteinuria is probably the variable with most evidence of an association with progression in dogs as well as in cats and people (Zoja *et al.*, 2015; Chakrabarti *et al.*, 2012; Li *et al.*, 2010; Syme *et al.*, 2006; Jacob *et al.*, 2005; Peterson *et al.*, 1995), The exact role of proteinuria in the pathogenesis and progression of canine CKD is uncertain, but recent evidence in canine medicine suggests that proteinuria may be a cause of tubulointerstitial damage rather than only a consequence of glomerular or tubular dysfunction, as shown in people (Benali *et al.*, 2013; Vilafranca *et al.*, 1995; Eddy & Michael, 1988). Excess filtered protein results in increased proximal tubular cell pinocytosis of proteins, which in turn may result in cellular damage because of swelling and rupture of lysosomes and increased production of pro-inflammatory mediators (Benali *et al.*, 2013; Vilafranca *et al.*, 1995; Bertani *et al.*, 1986). Proteinaceous casts may also obstruct tubuli and contribute to intrarenal damage.

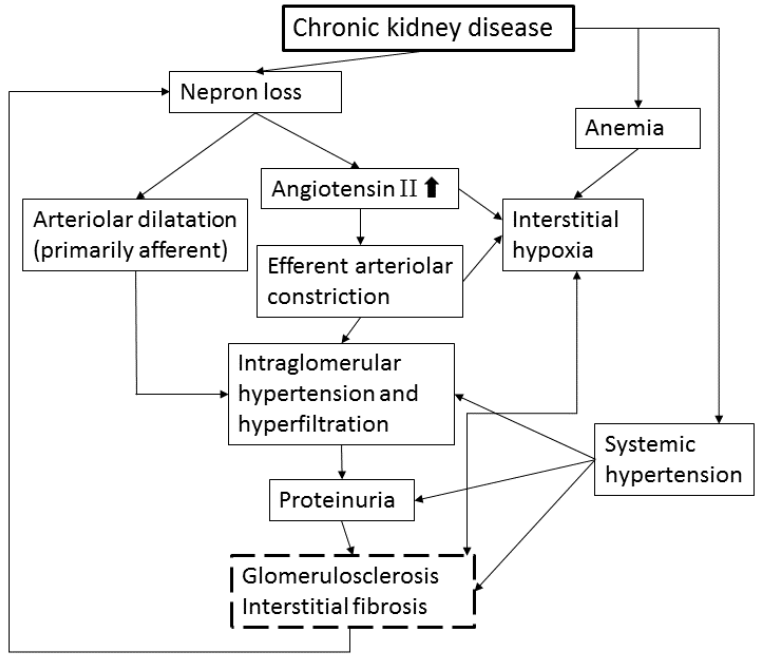


Fig 4. Simplified model of pathophysiological factors contributing to progression and accumulating fibrosis in chronic kidney disease.

The exact aetiology of CKD may influence rate of progression in dogs and people, as may superimposed clinical or subclinical AKI, "acute-on-chronic disease" (Venkatachalam *et al.*, 2015; Polichnowski *et al.*, 2014; Williams *et al.*, 1988). Other factors associated with rate of progression are systemic and glomerular hypertension (Lash *et al.*, 2009; Finco, 2004; Jacob *et al.*, 2003; Tozawa *et al.*, 2003), intrarenal hypoxia (Tanaka *et al.*, 2014; Mimura & Nangaku, 2010) and the mineral and bone disease associated with CKD, CKD-MBD (Lippi *et al.*, 2014; Natoli *et al.*, 2013). Recently, dehydration and osmotic stress were recognised as potential driving forces of progression in people with CKD (Gil *et al.*, 2018; Clark *et al.*, 2016). Reactive oxygen species generation, or "oxidative stress", may play a central part in the pathogenesis of progression of CKD of both dogs and people, potentially involving several of the aforementioned factors (Kogika *et al.*, 2015; Xu *et al.*, 2015; Brown, 2008).

Tissue fibrosis

Parenchymal destruction and fibrosis is the final pathway in the pathogenesis and the common end-point of progressive CKD, regardless of aetiology. Fibrosis, which may affect all parts of the kidney, is called glomerulosclerosis when affecting the glomerular compartment, and tubulointerstitial fibrosis when affecting the tubulointerstitial compartment. Because of the intimate connections between these functional parts, disease in one compartment negatively affects the other. An example of this is the tubulointerstitial damage that may arise secondary to glomerular proteinuria (Lazzara & Deen, 2007).

The division of renal fibrosis pathophysiology into four phases has been suggested, based on studies predominantly in rats and mice (Eddy, 2000). Recent studies have shown similar histopathologic patterns in canine progressive CKD (Benali *et al.*, 2014; Aresu *et al.*, 2011). The first phase, "cellular activation and injury", is characterized by recruitment of inflammatory cells and appearance of myofibroblasts. In the second "fibrogenic signalling" phase, the number of myofibroblasts increase. During the third, "fibrogenic" phase, accumulation of ECM occur because of an imbalance between matrix synthesis and degradation. In the fourth, "renal destruction" phase, ECM accumulation and nephron destruction is seen. The start of the fourth phase was described as the point of irreversibility, because of permanent destruction of renal structural elements. These phases have been studied in animal models after injury at one point in time, but in spontaneous disease many or all of these phases may be observed histologically at the same time (Eddy, 2000).

Synthesis, degradation and renal accumulation of ECM proteins and induction of proteases and other ECM-remodelling enzymes all contribute to development of renal interstitial fibrosis and disease progression (Eddy, 2014).

Renal tubular epithelial cells are thought to play a role in both the initial lymphocyte recruitment and in the fibrogenic phases as progenitors to the increasing population of myofibroblasts in the canine kidney (epithelial-to-mesenchymal transition (Benali *et al.*, 2014). The induction and proliferation of myofibroblasts is thought to represent a central event in the initiation and propagation of fibrosis in dogs and people (Benali *et al.*, 2014; Genovese *et al.*, 2014).

3.3 Prognosis

The long-term prognosis for dogs with a diagnosis of CKD is often referred to as grave. However, the outcome is considerably variable between dogs, partly because of differences in progression rates and partly as a result of the definition of canine CKD. Some dogs that are given a diagnosis of CKD at an early stage, do not develop progressive CKD. Also, a CKD diagnosis can be based on persistent (>3 months) renal proteinuria, which may later resolve. Consequently, canine CKD, as currently defined in veterinary medicine, may be either static (non-progressive) or active (progressive). When the disease is progressive, most dogs proceed to end-stage disease and death, but rate of progression is variable both within and between individuals (Finco *et al.*, 1999). It is difficult to predict the rate of progression in individual dogs. The fact that progression presumably is neither linear nor predictable is also recognised in human medicine (Onuigbo & Agbasi, 2014).

3.4 Clinical signs

Dogs with CKD may not show any clinical signs of disease. Because of the large reserve capacity of kidneys, clinical signs do not ensue until considerable loss of renal mass has occurred. Consequently, renal compromise is substantial at the time of diagnosis in many dogs. Clinical signs that may be present are PU/PD, weight loss, stunted growth, inappetence and vomiting, depending on degree of renal function loss.

3.5 Systemic consequences

When nephrons are lost and multiple renal functions decline, systemic consequences are inevitable. Some of the factors have been associated with CKD

progression, as mentioned above, and others (e.g. urinary tract infection, UTI) may be either a cause or a consequence of CKD.

The cardiovascular system and the kidneys interact extensively in both health and disease. Haemodynamic stability (including circulating volume and vasomotor tone), is controlled by the kidneys and the cardiovascular system in concert and disease of one organ system may lead to disease of the other. In people, cardiovascular pathology is a significant cause of morbidity and mortality in CKD patients (Di Lullo *et al.*, 2015; McCullough & Verrill, 2010). It has been shown in people that the age-specific risk of cardiovascular events was 17-fold higher in individuals with a low estimated GFR (eGFR; <15 ml/min/1.73 m^2) compared to those with a higher eGFR (DuBose, 2007). The term "cardiorenal syndrome" is used in human medicine to describe the pathological interplay between the two organ systems (Braam *et al.*, 2014; Brisco & Testani, 2014). Clinical importance of this interplay in canine patients remains unknown and the specific interactions may differ from those in humans, as well as between animal species. Therefore, the term cardiovascular renal axis disorders (CvRD) has been proposed for use in companion animal medicine (Pouchelon *et al.*, 2015). Disease or dysfunction of the cardiovascular system secondary to kidney disease was further characterised as $CvRD_k$. Direct evidence for the existence of $CvRD_k$ in dogs is scarce, but detrimental interactions between the kidney and the cardiovascular system have been documented. Examples are hyperkalaemia, anaemia, hypertension and hyper- or hypovolemia. In addition, decreased renal excretion of potentially toxic substances in individuals with kidney disease may negatively affect the cardiovascular system.

Biomarkers may be used to evaluate cardiovascular compromise in dogs with CKD. Cardiac-specific troponin I (cTnI) is released into the circulation in response to myocyte injury, and amino-terminal pro-B-type natriuretic peptide (NT-proBNP) in response to myocardial wall stretch. This stretch may be caused by increased arterial blood pressure or increased circulating blood volume, leading to intracardiac pressure overload (Maeda *et al.*, 1998). Higher serum and plasma concentrations of both cTnI and NT-pro-BNP have been documented in dogs with azotaemia, compared to healthy dogs (Raffan *et al.*, 2009; Schmidt *et al.*, 2009; Sharkey *et al.*, 2009; Boswood *et al.*, 2008; Porciello *et al.*, 2008). If these high biomarker concentrations are indications of $CvRD_k$ or of accumulation due to decreased GFR in dogs, has not previously been studied. Circulating blood volume may decrease or increase, particularly in later stages of CKD, or if pronounced proteinuria and nephrotic syndrome are present (Klosterman *et al.*, 2011). Increased cTnI concentrations in people with CKD

have been documented, but the reason for cTnI leakage from myocardial cells is not known (Freda *et al.*, 2002).

Anaemia is a common complication of canine CKD (Kogika *et al.*, 2015). Relative deficiency of EPO, uraemia-induced erythrocyte membrane fragility, and gastro-intestinal blood loss have all been suggested to contribute to anaemia development (Crivellenti *et al.*, 2017; Fiocchi *et al.*, 2017). Weakness, lethargy and renal medullary hypoxia are possible clinical consequences.

Systemic hypertension occurs in canine CKD. The healthy canine kidney is capable of autoregulation through tubuloglomerular feedback and myogenic properties of vessel walls (Just *et al.*, 1998; Herbaczynska-Cedro & Vane, 1973). This way, ideal intrarenal blood pressure is maintained throughout a wide range of renal perfusion pressures. In the diseased kidney however, autoregulation capability may decrease and increased systemic blood pressure may further damage the glomerulus and contribute to glomerular protein loss.

As renal function declines, the ability to excrete phosphate decreases. Adaptive change in concentration of fibroblast growth factor 23 (FGF-23) is necessary in order to maintain the concentration of phosphate within the reference interval. Increased FGF-23 and parathyroid hormone represent early indicators of CKD-MBD, or secondary renal hyperparathyroidism (Harjes *et al.*, 2017). Possible clinical consequences of CKD-MBD are osteodystrophy, metastatic tissue calcification and CKD progression (Shipov *et al.*, 2018; Lippi *et al.*, 2014; Olgaard *et al.*, 1984). Additional systemic consequences of CKD include acid-base and electrolyte disturbances, inappetence, nausea, gastro-interstinal pathology and urinary tract infection.

4 Diagnosis of CKD

A clinical diagnosis of canine CKD requires documentation of decreased renal function, structural renal pathology, or both. There is also a temporal aspect to the diagnosis, in that any abnormalities detected must have persisted for a period of time, usually three months or more, as mentioned in section 1 (Polzin, 2017). This temporal aspect is assured either by documenting persistence of abnormalities for at least the time period specified, or by documenting abnormalities that are by definition chronic (pronounced fibrosis).
Every clinical diagnostic evaluation starts with obtaining a case history and performing a physical examination. These crucial initial steps constitute the basis for determining pre-test probability of disease, unless the diagnosis is immediately obvious. Knowledge of the occurrence of different diseases in dog populations is clinically helpful, contributing value in the diagnostic thought process.

4.1 Epidemiology of kidney disease in dogs

Incidence- or mortality rates of CKD in dogs have not previously been reported. Incidence relates to new occurrences of disease over a period of time (Fig 5), and are usually expressed as a proportion (cumulative incidence) or a rate. The incidence rate (IR) refers to the number of new cases that develop per unit of time. Similarly, mortality rate (MR) refers to the number of deaths that occur per unit of time. Calculation of incidence (and mortality) rates requires knowledge of the population at risk. This information is often not available, and therefore, prevalence constitutes the epidemiological term most commonly estimated in canine medicine.

Prevalence refers to the proportion of individuals that are affected by disease at any point in time, in a defined group of individuals (Fig 5). Prevalence calculations are often performed in “convenience” populations, such as the total

number of dogs that visited one or more veterinary medical institutions over a specified duration of time. As a result, external validity of the results may be limited (referral bias) (Bartlett *et al.*, 2010).

Technical developments have led to an increasing number of information registers and databases where information is gathered, which is potentially useful for epidemiological studies. If the information in a database was not collected with the explicit purpose of research, it is referred to as secondary rather than primary (Emanuelson & Egenvall, 2014). In secondary databases, sample size can be considerable, representativeness of the population high and risk of bias, such as non-response or recall bias, low (Roos L L, 1990). The main limitation is suboptimal data resolution. Validation of the chosen data source for the intended use should be performed (Emanuelson & Egenvall, 2014).

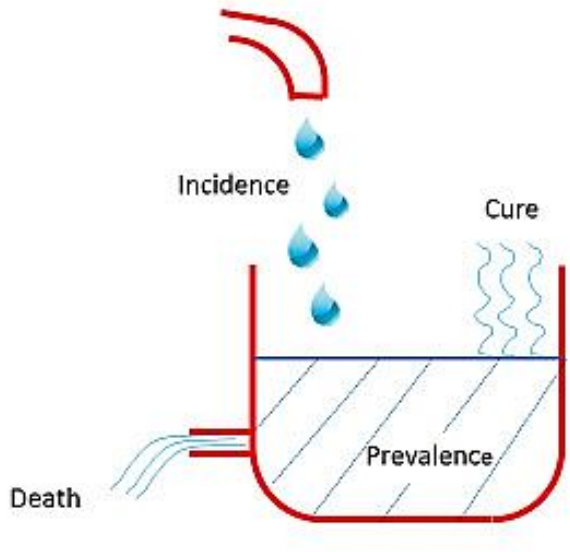


Fig 5. Schematic illustration of epidemiological concepts. Prevalence – proportion of affected individuals at one point in time. Incidence - new occurrences of disease over a specified period of time. Prevalence decreases when individuals regain health, or when they die, and thereby leave the population. Treatment of disease may delay death (increase prevalence) or facilitate cure (decrease prevalence). Disease chronicity is another factor that influences prevalence, because the longer the time that an individual is affected, the more individuals will have the disease at one point in time (increased prevalence). Thus, prevalence is useful for determining the burden of a disease on a population, while incidence provides useful information regarding risk of contracting disease and changes in disease occurrence over time.

4.2 Assessment of kidney structure (morphology)

Structural damage to the kidneys may be assessed macroscopically at surgery, or at post mortem examination. Different diagnostic imaging modalities; radiography, ultrasound, computed tomography (CT) and magnetic resonance imaging provide less invasive options for macroscopic evaluation of renal morphology in clinical patients. With the use of ultrasound, the size, shape, and parenchymal architecture of the kidneys may be assessed (Fig 6). Echogenicity of the canine renal cortex is similar to that of the liver and slightly less echogenic than that of the spleen. The medulla is less echogenic than the cortex and the cortico-medullary border is normally readily identified.

Diagnostic imaging modalities are effective in diagnosing focal morphologic abnormalities such as mass lesions, fluid accumulation, renal cysts or agenesis, but for definitive diagnosis of diffuse parenchymal disease, histopathology is needed. This may be performed either by obtaining renal biopsies from a living dog, or as part of a post-mortem examination. Renal biopsy should only be performed when results are likely to be of value in case management, and when no contraindications, such as uncontrolled hypertension, coagulopathy or severe azotaemia, are present.

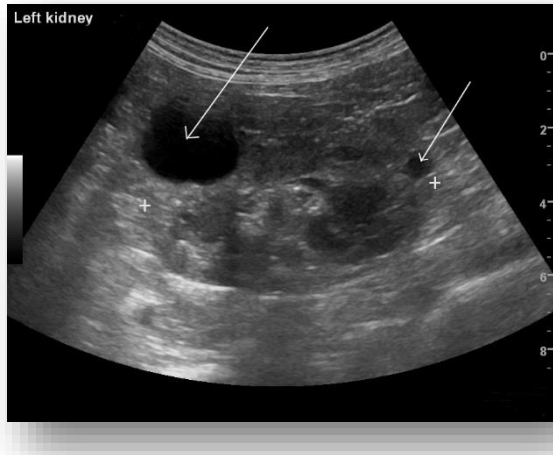


Fig 6. Image acquired by renal ultrasound, showing an example of structural abnormality; multiple cysts in the left kidney (arrows) in one of the dogs with polycystic kidney disease, included in studies II-IV. (Courtesy of Chiara Mattei).

4.3 Assessment of global function (GFR)

Measurement of GFR is generally considered the most sensitive index of functional renal mass and the best indicator of global renal function. The GFR

may be calculated by different methodologies. The golden standard method in human medicine is urinary clearance of inulin, but this method is not practical in the clinical setting (Von Hendy-Willson & Pressler, 2011). Plasma clearance studies negate the need for timed urine collections and, instead, rely on timed blood collections (Heiene & Moe, 1998). Limited sampling methods for clinical use have been proposed for both dogs and cats (Finch *et al.*, 2011; Heiene & Moe, 1999).

With image-based techniques, renal uptake of a filtered marker can be measured. Two examples of imaging techniques that have been validated for GFR measurement in dogs are scintigraphy and dynamic computer tomography (Chang *et al.*, 2011; Krawiec *et al.*, 1988). Contrast-enhanced computer tomography has been described to underestimate global GFR compared to both scintigraphy and clearance of iohexol in healthy dogs (O'Dell-Anderson *et al.*, 2006).

Results from any GFR-assessment technique represents the GFR accomplished by the kidney(s) at the very time of measurement (in relation to the normalisation variable), not the GFR that "could" be obtained in an optimal situation, which is sometimes mistakenly assumed. Any measured value is the combined result of pre-renal, renal and post-renal factors affecting GFR. Hypotension may decrease GFR because of decreased hydrostatic pressure in the glomerular capillary tuft, and urinary obstruction increases hydrostatic pressure in Bowman´s capsule, thus decreasing GFR. Another important clinical feature is the chronicity of any GFR decrease. The obtained GFR value may represent a chronic, successive decrease, or an acute decrease or a combination of both (Cowgill *et al.*, 2016; Grauer, 1998). These aspects are important in the clinical assessment of an individual case.

Also, importantly, GFR in a clinically stable dog with progressive CKD at any point in time represents a balance between nephron hypertrophy/ hyperfiltration and nephron destruction. Thus, if renal function is perceived as stable in a dog over time, this may represent truly stable function, but it may also represent a balance between nephrons that are destructed and nephrons that are over-working in order to maintain global function (Finco *et al.*, 1999). Measurement of GFR conveys no information regarding which intrarenal processes that are occurring at a specific point in time. Rather, the net effect of these processes is measured.

In clinical practice, GFR is most often estimated by measuring concentrations of circulating indirect biomarkers, such as creatinine, but techniques for GFR measurement, for example clearance studies or scintigraphy, are also sometimes used.

4.3.1 Scintigraphy

With renal scintigraphy, a two-dimensional picture of the urinary tract is produced, in that the uptake of an intravenously injected radiopharmaceutical (Technetium-99m-dietylene-triaminepentaacetic acid, ^{99m}Tc-DTPA) over time is detected by a gamma camera that counts radioactive emissions. The pharmaceutical (DTPA) meets the criteria stipulated for a good marker of GFR, and its uptake by filtration in the kidney, as a fraction of the injected activity of ^{99m}Tc-DTPA, is directly related to GFR (Gates, 1982). Several techniques exist for measurement of GFR following scintigraphy. Two examples are the integral (or Gates´) method and the plasma volume method (Kampa *et al.*, 2007; Gates, 1982). Calculation of GFR is performed for each kidney separately. Renal scintigraphy with 99mTc-DTPA has been evaluated and found useful for GFR-measurement in dogs (Krawiec *et al.*, 1988).

In order to compare GFR between individuals, it must be normalised to some measurement related to body size. In human medicine, GFR is often normalised to body surface area (BSA). The use of BSA as a normalising factor has been questioned in veterinary medicine, especially for drug dosing (Price & Frazier, 1998). In dogs, GFR is usually normalised to body weight (BW, integral method) although methods of normalisation to plasma volume also have been recommended (Westgren *et al.*, 2014; Kampa *et al.*, 2007). From the physiological standpoint, because GFR changes in accordance with volaemic status of the animal (Chew & Gieg, 2006), normalisation of GFR to plasma volume is more reasonable than BW. Receptors for detection of volume status are present in the intravascular space and not in the interstitial fluid space, and therefore plasma volume is conceptually superior for normalisation of GFR compared to the extra-cellular fluid volume (Peters *et al.*, 1994). Reference intervals for GFR in dogs remain poorly defined and vary with methodology, normalisation method and individual characteristics of the animal (Von Hendy-Willson & Pressler, 2011). Bodyweight has been shown to be associated with GFR measured by plasma clearance of iohexol in a study of 118 dogs (Bexfield *et al.*, 2008). Therefore, slightly different reference intervals were proposed for the different BW quartiles (2-12, 13-25, 26-31 and 32-70 kg, respectively).

4.3.2 Circulating biomarkers

For a long time, creatinine has been the circulating biomarker of choice for a quick and simple, relatively non-invasive, assessment of GFR. Creatinine concentration is also used for staging of CKD (section 4.3.3). Other markers of decreased GFR, for example cystatin C and symmetric dimethyl arginine (SDMA) have been studied. Recently, SDMA was made commercially available

and is now extensively used for evaluation of renal function in dogs. The value of adding other circulating markers to creatinine in the clinical work-up of dogs with suspected CKD is not known.

Creatinine

Creatinine in the circulation originates mainly from degradation of creatine and creatine phosphate in muscle tissue, but a small part may originate from alimentary supply (Harris *et al.*, 1997). Creatinine is released into the circulation at a stable rate proportional to muscle mass, cleared by glomerular filtration and not reabsorbed. Urinary elimination of creatinine is relatively constant over time in an individual, but may differ greatly between individuals (Jergens *et al.*, 1987; Barsanti & Finco, 1979). Minimal tubular secretion occurs in male dogs (O'Connell *et al.*, 1962) but this is of little significance (Watson *et al.*, 2002; Labato & Ross, 1991). Therefore, creatinine possesses many of the properties of an optimal biomarker of GFR.

Methods of creatinine concentration measurement include the Jaffe reaction and enzymatic methods. Enzymatic methods are preferred because of the interaction of non-creatinine chromogens with the Jaffe reaction (Delanghe & Speeckaert, 2011). Unfortunately, no international standardisation of creatinine analysis has been performed despite differences in assay performance (Ulleberg *et al.*, 2011; Braun *et al.*, 2008). In addition, reference ranges differed extensively between laboratories (Ulleberg *et al.*, 2011). Creatinine concentration is generally higher in large breeds (Finch & Heiene, 2017; Middleton *et al.*, 2017; Misbach *et al.*, 2014; Medaille *et al.*, 2004; van den Brom & Biewenga, 1981), and different reference intervals for different BW quartiles have been suggested. Creatinine concentration is higher in greyhounds (sighthounds) than in many other breeds (Dunlop *et al.*, 2011; Zaldivar-Lopez *et al.*, 2011; Feeman *et al.*, 2003). Published data concerning the effect of age on creatinine concentration are somewhat inconsistent but it seems that creatinine concentration decreases in the first days after birth, then remains stable for two months, after which it increases until approximately one year of age (Wolford *et al.*, 1988). Thereafter, it remains comparably stable until a high age (Fukuda *et al.*, 1989). In one study, there was no difference between creatinine concentrations in young compared to old Beagle dogs (Vajdovich *et al.*, 1997).

Symmetric dimethyl-arginine

A new potential circulating biomarker of GFR is SDMA. It is derived from intracellular protein metabolism and mainly cleared by glomerular filtration (McDermott, 1976; Kakimoto & Akazawa, 1970). In human medicine, SDMA

concentration is not widely used clinically as a marker of GFR, but its diagnostic value has recently been studied (El-Khoury *et al.,* 2016; Kielstein *et al.,* 2011; Tutarel *et al.,* 2011). A liquid chromatography-mass spectrometry assay of SDMA has been validated (Nabity *et al.,* 2015). Other assays for SDMA measurement in dogs are currently available and extensively promoted on the veterinary market. There is, however, very limited information available about the diagnostic performance of SDMA concentration in plasma or serum as a marker of GFR in dogs (Nabity *et al.,* 2015). Age, sex and BW have been described not to influence SDMA concentrations in dogs (Moesgaard *et al.,* 2007; Pedersen *et al.,* 2006). The SDMA concentration has been shown to be stable in samples stored for seven and 14 days at 20° and 4°, respectively, and after three freeze-thaw cycles (Nabity *et al.,* 2015).

Cystatin C

Cystatin C, a low-molecular-weight (13 kDa) cysteine protease, is produced at a stable rate by all nucleated cells and cleared by glomerular filtration (Jacobsson *et al.,* 1995; Abrahamson *et al.,* 1990). Neither age nor BW seem to significantly affect circulating cystatin C concentration in people or in dogs (Miyagawa *et al.,* 2009; Wehner *et al.,* 2008; Finney *et al.,* 2000; Norlund *et al.,* 1997). In one study, cystatin C concentrations were marginally higher in young and old dogs than in mature adults, but the difference was not large enough to warrant different reference intervals in different age groups (Braun *et al.,* 2002).

Cystatin C is routinely used in equations for calculation of eGFR in people, either alone or together with creatinine (Grubb *et al.,* 2014; Larsson *et al.,* 2004). In dogs, cystatin C concentration in serum or plasma has been evaluated as a marker of GFR using different analytical methods and study designs, and this biomarker is considered potentially useful (Miyagawa *et al.,* 2009; Wehner *et al.,* 2008; Almy *et al.,* 2002). Exogenous corticosteroid administration can increase cystatin C concentrations in dogs, in contrast to endogenous overproduction in hyperadrenocorticism (Muñoz *et al.,* 2017; Marynissen *et al.,* 2016). Hyper- and hypothyroidism has been shown to affect cystatin C concentrations, at least in cats and people (Ghys *et al.,* 2016; Jayagopal *et al.,* 2003).

Diagnostic test properties relevant to biomarkers of GFR

Many aspects of biomarkers of disease need consideration. The most commonly communicated properties of a diagnostic test are sensitivity and specificity. When results of a diagnostic test are interpreted, however, sensitivity and specificity have no direct diagnostic meaning. Predictive values are more useful

in this respect, but unfortunately differ considerably between individuals with different pre-test probabilities, and thus cannot be used as fixed properties of a certain test (Gallagher, 2003).

In contrast, a diagnostic test property that is stable (not critically dependent on disease probability), and therefore may be conveniently applied across individuals and populations, is the likelihood ratio (LR) (Dujardin *et al.*, 1994). Calculated from sensitivity and specificity, it summarizes information from both terms and provide the discriminatory power of the test. These ratios are presented as positive (LR+) and negative (LR-) ratios, where LR- is a number between 0 and 1, and LR+ a number ≥ 1. Pre-test probability of disease is multiplied with the LR to provide a post-test probability of disease. Diagnostic tests with LR- below 0.1 or LR+ >10 modify the pre-test probability in a highly useful way (Gallagher, 1998).

For continuous tests like the indirect biomarkers of GFR, LRs may also be defined for multiple intervals of the test result. With the use of such *interval LRs*, more information from a test result is gained, compared to when continuous results are dichotomised as positive/high or negative/normal.

In veterinary medicine, cross-sectionally derived, population-based, reference intervals are used in the interpretation of diagnostic test results. The total variation between results from a group of individuals (used to create a population-based reference interval) consists of the sum of pre-analytical, analytical, inter-individual (CV_g) and intra-individual (CVi) variation. The CV_g contributes with a large part of the width of population based reference intervals for some variables. An example of such a variable (with $CV_g >> CV_i$, or a high individuality) is creatinine. The concentration (of for example creatinine) in an individual over time may reside in the middle, towards the upper or lower reference limits, or even outside a population-based reference interval (Fraser, 2004). This markedly affects overall diagnostic performance of the biomarker. Assigning different reference intervals to relevant sub-groups, for example breed (stratification), may improve utility of such a test. Alternatively, a longitudinally derived reference change value (RCV), determined based on the analytical and biological variation of a variable, may be used (Walton, 2012). This way, a significant change (possibly indicating pathology) between two consecutive measurements can be detected even if both results are within a broad population based reference interval.

4.3.3 Staging of canine CKD

Staging of CKD provides an opportunity for a comparable description of clinical cases and makes the development of specific recommendations regarding diagnostic tests and treatment options for patients in different stages of disease possible. The international renal interest society (IRIS) has developed a staging system for CKD in dogs (IRIS, 2016). It is based on "stable" (i.e. in steady state) fasting serum creatinine concentration, in order to make staging possible in virtually every clinical environment. Staging can only be done once a definitive diagnosis of CKD has been established.

Stage 1 dogs are those with a stable creatinine concentration <125 µmol/L. Stage 2 includes dogs with a creatinine concentration between 125-180 µmol/L, stage 3 those between 181-440 µmol/L and stage 4 dogs those with a creatinine concentration >440 µmol/L (IRIS, 2016). Substaging of CKD is performed by including results from blood pressure measurement and persistent renal proteinuria assessment. The lack of standardisation of creatinine assays and the variation in creatinine concentration between dogs of different bodyweights and breeds noted above, as well as blood pressure equipment and procedure variations, might leave staging of canine CKD a somewhat imprecise activity.

4.4 Urine analyses

4.4.1 Routine urine analyses

Urine has long been used for diagnostic purposes in the medical profession. Already in the 17th century, "piss-prophets" diagnosed all sorts of diseases by looking at the colour of, and sometimes tasting, urine from their patients. Urine represents a unique diagnostic fluid because it may be obtained non-invasively in large amounts. Also, it represents a fluid sample produced by and acquired from the kidney, and as such is invaluable for renal diagnostic procedures. Despite this, urine analysis is sometimes underutilised by veterinarians. Urine may be collected by spontaneous voiding, catheterization or cystocentesis. Results of any diagnostic tests are interpreted in light of collection method as well as of storage time and temperature, if urine analysis is delayed. Collection of urine is preferably performed before administration of fluids or other types of medication. Initially, colour and appearance of urine are subjectively assessed, thereafter routine analyses are performed. These usually consist of dipstick analysis, urine specific gravity (USG) and sediment examination.

If pathological proteinuria is suspected, quantification may be performed by determining the urine protein-to-creatinine ratio (UPC). Values of UPC from

random voided urine samples from dogs are correlated with total 24-hour protein excretion (Center *et al.*, 1985; Grauer *et al.*, 1985). International reference intervals and treatment thresholds exist and are in use, but presently, there is no international standardisation of UPC measurements. Pathologically high amounts of protein in the urine may be characterized as pre-renal, renal, or post-renal. Renal proteinuria may be glomerular (filtration barrier leakage) or tubular (tubular cell dysfunction), or both. If proteinuria is renal and persistent (>3 months), CKD is present according to the current CKD definition. Biological variation of UPC is of clinical importance and should be taken into account both in diagnosis and in monitoring outcome of treatment (Nabity *et al.*, 2007).

4.4.2 Urinary proteomics

As noted in section 4.3, the kidneys possess a large functional reserve capacity and can compensate for large decreases in renal functional mass. Thus, a reduction in GFR does not occur until the compensatory adaptation of the kidneys fails. Therefore, even GFR measured with clearance studies or scintigraphy is a comparably insensitive indicator of decreasing renal function in CKD. Detection of the fibrotic process itself could possibly provide an earlier opportunity for diagnosis. Currently, the only way of definitively diagnosing interstitial fibrosis clinically is by renal biopsy. This procedure is costly, invasive and not without risk. The development of non-invasive, fibrosis-specific biomarkers, reflecting morphological tissue change at an early stage of CKD, could be a major breakthrough for earlier and more specific diagnosis of canine CKD.

With the use of proteomics, multiple urinary proteins and peptides can be identified in a non-invasive manner. The urinary proteome, although less complex than that of plasma, contains thousands of proteins and peptides (Kentsis *et al.*, 2009; Coon *et al.*, 2008). Urine as a source of proteomic biomarkers is favourable since it contains a lower dynamic range of analytes compared to plasma. The urinary proteome is also thought to be highly stable because of minimal to no ongoing proteolysis in contrast to the situation in plasma (Mischak *et al.*, 2010b).

Proteins and peptides from the urinary tract are either secreted from tubular cells or originate from epithelial cells shed from any part of the urinary tract (including urethra and the genital tract if spontaneously voided urine is used). About 70% of the human proteome in health originated from the urinary tract itself in one study (Pieper *et al.*, 2004).

Low molecular weight proteins and peptides in urine are either freely filtered through glomeruli from the blood, or they may originate from the urinary tract itself. Accumulated data indicate that approximately 80% of urinary peptides originate from the kidney (Krochmal *et al.*, 2018). Many of the sequenced urinary peptides represent fragments of collagen.

Diagnostic use of urinary proteomics-based classifying models represents a novel strategy that may substantially contribute to clinical management of CKD, particularly given the non-invasiveness of urine collection and the stability of the urinary proteome (Mischak *et al.*, 2010a).

Capillary electrophoresis coupled to mass spectometry

Urinary proteome analysis by capillary electrophoresis coupled to mass spectrometry (CE-MS) enables robust and reproducible analysis of LMW proteins and peptides in people. The method provides fast (<1 hour) separation with high resolution at a low cost (Hernandez-Borges *et al.*, 2004; Neususs *et al.*, 2002). The CE-MS technology does not include tryptic digestion of urinary proteins, and therefore allows analysis of naturally occurring peptides. This subfield of proteomics is called peptidomics. The CE-MS urinary peptidome analysis requires depletion of proteins with a molecular weight >20 kDa because larger proteins will clog the capillary. Hence, with the CE-MS approach, whole proteins cannot be assessed. Peptides that are bound to proteins are also removed in this step and therefore not assessed. There are, however, several advantages of focusing on peptides instead of proteins. The stability of the urinary peptidome is significantly higher than that of the urinary proteome, (Good *et al.*, 2010) (Good et al., 2010) probably because peptides are in themselves degradation products (Klein *et al.*, 2016; Theodorescu *et al.*, 2006). The human urinary peptidome is stable for three days at 4°C, several years at -20°C and after repeated freeze-thaw cycles (Mischak *et al.*, 2013). Analysis of the urinary peptide content has also shown a higher reproducibility than proteomics analyses, presumably because in peptidomics analyses, mass spectrometry can be performed without tryptic digestion (Mischak *et al.*, 2013).

Using CE-MS, a classifying model consisting of 273 urinary peptide biomarkers has been developed and subsequently validated in a separate cohort of people (Good *et al.*, 2010). This model, called CKD273, was shown to differentiate healthy individuals from those with CKD, irrespective of underlying aetiology. . The diagnostic potential of the CKD273-model has been confirmed in further, cross-sectional and prospective, studies (Roscioni *et al.*, 2013; Zurbig *et al.*, 2012; Alkhalaf *et al.*, 2010). The CKD273-model is currently applied for patient stratification in a multicentric randomised clinical trial, the PRIORITY trial (Pontillo & Mischak, 2017). The aim of this trial is to

investigate the potential benefit of early detection (by the CKD273-model), and spironolactone treatment, of human diabetic nephropathy.

For diagnostic purposes, only peptide physiochemical properties (such as molecular mass and migration time) are necessary. Identification of differentially expressed, naturally occurring, peptides may, however, facilitate further pathophysiological insight. Over the last decade, urinary peptidomics has provided new information regarding human renal pathophysiological mechanisms. Naturally occurring urinary peptides, such as those identified with CE-MS, result from proteolytic activity in the body. Changes in extracellular protease activity may be responsible for changes in the urinary peptidome, which in turn may reflect presence (or progression) of a specific disease (Candiano *et al.*, 2006).

5 Aims of the thesis

The general aim was to increase knowledge regarding pathophysiologic mechanisms and early diagnosis of canine CKD, by identifying dogs with increased risk of disease and by exploring the use of various biomarkers in blood and urine.

The specific aims were as follows:

- To use data from a large insurance company database to estimate morbidity and mortality related to kidney disease in a Swedish population of insured dogs.

- To investigate possible associations between plasma concentrations of cTnI and NT-proBNP, respectively, and patient characteristics, GFR, PVF/kg, SBP, selected hematologic and biochemical variables and echocardiographic measurements in dogs with stable CKD and in healthy dogs.

- To compare the overall diagnostic accuracy for SDMA and cystatin C compared to creatinine, for detection of decreased GFR in clinically stable dogs with a diagnosis of, or a strong suspicion of, CKD. Also, to evaluate if combining SDMA, cystatin C, or both, with creatinine increases diagnostic value compared to the use of creatinine alone.

- To evaluate if CE-MS-based urinary peptidome analysis can discriminate healthy dogs from dogs with CKD by constructing classifying models and validate them in a separate group of dogs. Also, to identify peptides included in the discriminating models.

6 Comments on materials and methods

6.1 Study populations and study design

6.1.1 Insurance database (paper I)

In paper I, data from a large Swedish insurance company was used to estimate kidney-related morbidity and mortality in Swedish insured dogs. The information in this database was previously validated against practice records (Egenvall *et al.*, 1998). We used data on all dogs in the Agria Animal Insurance database over a 12 year period (1995 through 2006) to investigate the incidence of kidney-related disease (KD) in Swedish insured dogs. The exact time each dog was insured was calculated from the day they were insured until either a diagnosis of KD, death, withdrawal from insurance, or the end of the study period. The common denominator in all calculations was "dog year at risk" (DYAR). One DYAR equals one year of insurance participation for one dog. As an example, if a dog was insured on January 1st, 1997 and died on July 1st, 2004, the contribution of this dog to the study was 7.5 DYAR. The large size of the study population also made stratifications according to breed, sex and age possible.

Calculations of incidence and mortality differences by breed were, however, restricted to breeds that contributed with more than a total of 10.000 DYAR, in an attempt to reduce bias. Incidence was calculated from data in the veterinary care insurance database and mortality was calculated from data in the life insurance database. The life insurance database contains data from dogs up to 10 years of age only, and consequently no information on mortality of KD after 10 years of age could be obtained. It was not possible to distinguish acute from chronic KD in this data. Consequently, results include both forms of disease.

6.1.2 Prospectively recruited dogs (papers II-IV)

Dogs with a diagnosis, or a strong suspicion, of CKD and healthy dogs were prospectively included at the University Animal Hospital in Uppsala between February 2012 and November 2016. Uppsala ethical committee approved the study and informed consent was collected from owners of all dogs included in the studies. The days of study inclusion of dogs were scheduled weeks ahead, in order to minimise any influence of for example acute-on-chronic disease that may be present when dogs are presented at the hospital. That way, we aimed to assure that GFR and other measured variables would reflect everyday homeostatic balance without influence of possible acute events. Owners were instructed to withhold food for 12 h before examinations. On the day of inclusion, a case history was obtained and every dog underwent a physical examination, repeated blood pressure measurements, urinary tract ultrasound, echocardiography and scintigraphy (Fig 7). Collection of blood and urine samples was performed, both for immediate analysis and for storage (-70°) until batch analysis. Dog-owners were present during all examinations, to keep dogs comfortable and calm. No dog was sedated.

In addition to urine samples from the 105 dogs included according to study protocol, urine from six healthy research beagles were also used in the peptidomics analyses of paper IV. These beagles were considered healthy due to a healthy appearance and according to results of blood and urine routine analyses. Renal histopathology by light microscopy was also performed and considered normal, in all six beagles.

Because of our attempt to include dogs in all stages of CKD, including those with mild, subclinical disease (stage 1), 14 of the included dogs with a suspicion of CKD, because of for example unexplained chronic PU/PD, could not be given a conclusive CKD diagnosis according to the current CKD definition. These dogs were classified as "inconclusive". All included dogs were free of other significant systemic or organ-related disease. The reason for including inconclusive dogs in paper III was that 1) association of biomarkers with GFR was of interest irrespective of a CKD diagnosis, 2) a population with a suspicion of kidney disease is more closely representative of the clinical population in which the tests evaluated in the study are to be used, than is a population with a diagnosis of CKD and 3) avoidance of a case control scenario is important in diagnostic studies. Similar to clinical trials, diagnostic test accuracy studies are at risk for systemic biases (Whiting *et al.*, 2013). There is evidence for effects on study validity of spectrum bias (case-control design) and partial or differential verification bias (using different reference tests, or a reference test that includes the index test), among others.

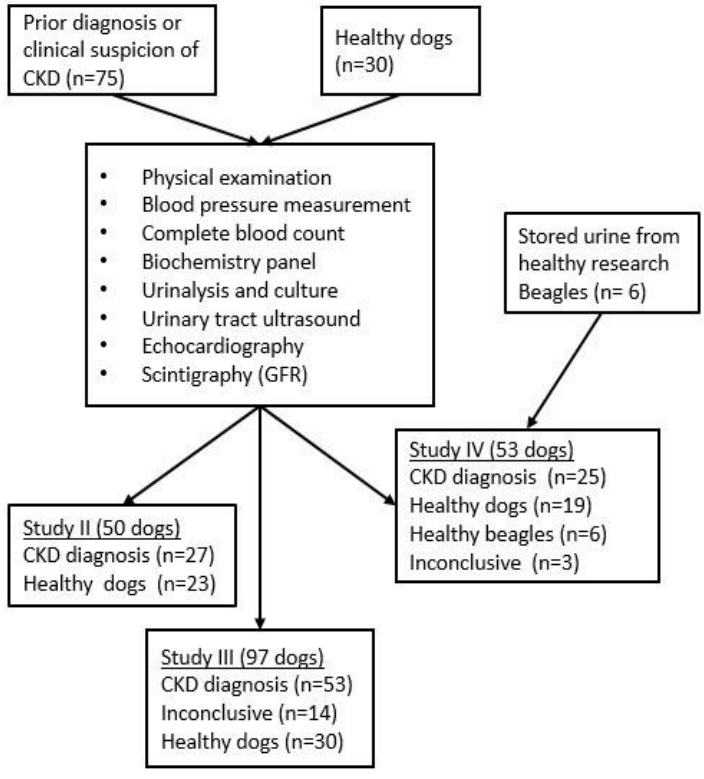


Fig 7. Flow-chart of included dogs in the PhD-project.

In the urinary peptidomics study (paper IV), no inconclusive dogs were used for model construction or validation, but three inconclusive dogs were included as examples of clinical patients for which a peptidomics analysis may be informative. Fifteen dogs with CKD and 15 healthy dogs comprised the training set (peptidome model construction). Twenty separate dogs (10 with CKD and 10 healthy dogs) comprised the independent validation set. Samples from dogs in the validation group (10+10 dogs) and from the three inconclusive dogs, were sent without identification and thus blinded to the researchers performing CE-MS analysis and the model validation process. Dogs with a confirmed diagnosis of CKD were staged according to the IRIS staging system described in section 4.3.3.

6.2 Examination procedures (papers II-IV)

6.2.1 Blood pressure measurement

Repeated systolic blood pressure (SBP) measurements were performed by high definition oscillometry (HDO) after 5-10 minutes of acclimatisation to the examination room. Dogs were held in standing position and minimally restrained. The cuff was applied at the tail-base in all dogs but one (which had no tail). In that dog, a front leg was used. At least 5-8 measurements were performed, and the mean of five SBP values with a variation of maximally ≈ 20% was calculated and used in statistical analyses (papers II-III).

6.2.2 Urinary tract ultrasonography

Ultrasonography of the entire urinary tract was performed in dorsal recumbency, according to a pre-specified protocol. Examinations were performed by experienced radiographers at the diagnostic imaging clinic of the University Animal Hospital. At the end of each examination, if possible, 10-40 ml of urine was obtained by cystocentesis.

6.2.3 Echocardiography

Echocardiographic examinations were performed in order to exclude primary heart disease and to assess intracardiac volume status. Dogs were examined in right and then left lateral recumbency by experienced ultrasonographers and standard views were obtained. Left atrial and ventricular measurements were made on three consecutive cardiac cycles, and the mean value from each dog was used in the statistical analysis. The echocardiographic unit used was iE33, Philips Ultrasound, Bothell, USA.

6.2.4 Sampling of blood and urine

Blood was collected by venipuncture into two and four ml tubes containing EDTA, or with no additive, and transferred to the University Animal Hospital laboratory for immediate routine analyses (CBC and biochemistry panel). Remaining serum and EDTA plasma was frozen in aliquots at -20°C for a maximum of 24 h, and thereafter transferred to storage at -70°C. Urine was collected by cystocentesis at the time of abdominal ultrasonography for most dogs. If cystocentesis was not possible, fresh voided urine was collected. After allocation of urine for immediate examination at the University Animal Hospital laboratory (dipstick, USG, UPC, sediment examination and urine culture),

remaining urine was aliquoted into plastic cryo tubes and stored at -70° C. Urine from the six research beagles was snap frozen at the time of sampling, and thereafter stored at -70° until batch analysis (paper IV).

6.2.5 Assessment of glomerular filtration rate

The scintigraphic examination was performed with dogs in lateral recumbency (Fig 8), as previously described (Westgren *et al.*, 2014; Kampa *et al.*, 2007; Kampa *et al.*, 2002). To obtain a dorsal view including kidneys and heart, the gamma camera was positioned vertically. Over six minutes, a dynamic acquisition was recorded at one frame per 10 seconds. Immediately after starting acquisition, a bolus of 70 mBq ^{99m}Tc-DTPA followed by a four ml saline flush was injected intravenously. In order to facilitate measurement of the distance between kidneys and gamma-camera, the camera was thereafter moved to a horizontal position above the dog, and a 30 second static lateral image was acquired. By subtracting pre- from post-injection syringe and catheter counts and correcting for radioactive decay, net injected radioactive counts were calculated. Commercial nuclear medicine software was used to calculate individual kidney GFR with both the integral method, in which GFR is normalised to BW, and the plasma volume method, in which GFR is normalised to plasma volume (Kampa *et al.*, 2007). The pre-defined cut-off value for decreased GFR with the plasma volume method, which was used in statistical calculations, was 30.8 ml/min/L (Kampa *et al.*, 2007).

Fig 8. Positioning of a dog during scintigraphic examination. The gamma-camera is positioned vertically, obtaining a six-minute dynamic acquisition. (Courtesy of Ina Larsson).

6.3 Analyses

6.3.1 Circulating biomarkers (papers II-IV)

N-terminal pro-B-type natriuretic peptide (paper II)

Frozen EDTA plasma was sent to Idexx Laboratories, Germany, for duplicate batch analysis of N-terminal pro B-type natriuretic peptide (NT-proBNP) with a commercially available canine ELISA (CardioPet proBNP, product number 2665, IDEXX Laboratories, USA). The lower limit of detection was 250 pmol/L.

Cardiac troponin I (paper II)

Frozen EDTA plasma was sent to the Department of Clinical Chemistry, Skene Hospital, Sweden. Batch-analysis of cardiac-specific Troponin I (cTnI) concentration in duplicate was performed using a high-sensitivity assay, Access AccucTnI+3 Troponin I assay, Beckman Coulter, USA. This assay was previously validated for use in dogs (Oyama & Solter, 2004). The lower limit of detection was 0.01 µg/L.

Creatinine (papers II-IV)

Serum concentration of creatinine was measured at the clinical chemistry laboratory at the University Animal Hospital in Uppsala, on the day of inclusion as part of the biochemistry analysis panel, according to study protocol. An enzymatic method was used, analysed on the Architect c4000 (Abbott Diagnostics) analyser. The pre-defined cut-off of creatinine for diagnosis of decreased GFR was 115 µmol/L.

Symmetric dimethyl-arginine (paper III)

Frozen serum was transported to IDEXX Laboratories for batch analysis of SDMA by the method offered commercially to companion animal clinics (enzyme immunoassay). Duplicate analysis was, for unknown reasons, not performed as requested. The pre-defined cut-off of SDMA for diagnosis of decreased GFR was 14 µg/L.

Cystatin C (paper III)

Frozen serum was transported to the Department of Clinical Chemistry, Uppsala Academic Hospital, for batch analysis of cystatin C concentration in duplicate.

A particle-enhanced immuno-turbidimetric (PETIA) assay (Gentian, Moss, Norway) was analysed using Architect c8200 (Abbott, USA). This assay had been previously validated for use in dogs (Monti *et al.*, 2012). An in-house validation confirmed acceptable linearity in the measurement range. This assay is not clinically used for dogs and, therefore, no pre-defined cut-off value for diagnosis of decreased GFR was available. A "defined" cut-off yielding a sensitivity equal to that of creatinine was therefore chosen for sensitivity and specificity analysis.

6.3.2 Capillary electrophoresis and mass spectometry (paper IV)

Analysis by CE-MS was performed as previously described (Mischak *et al.*, 2013) using a Beckman Coulter Proteome Lab PA800 capillary electrophoresis system online coupled to a microTOF II mass spectrometer (MS; Bruker Daltonic, Germany). Peptide abundance was normalised to naturally occurring urinary polypeptide standards in order to compensate for differences in USG between different urine samples.

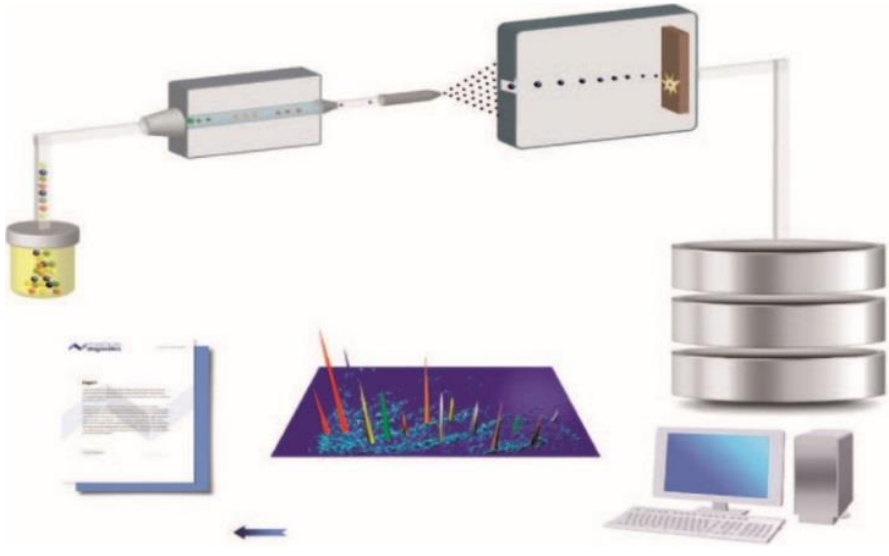


Fig 9. Urinary capillary electrophoresis coupled to mass spectrometry (CE-MS) work-flow. Urinary proteins are detected by CE-MS, where mass and relative abundance of each protein is analysed. All detected peptides are stored in a database, allowing further evaluation of peptides for diagnostic purposes. Reproduced with permission from Pontillo and Mischak. Urinary peptide-based classifier CKD273: towards clinical application in chronic kidney disease. Clinical Kidney Journal (2017) 10(2):192-201. Published by Oxford University Press on behalf of ERA-EDTA.

For peptide sequencing, liquid chromatography-MS/MS (Dionex Ultimate 3000 RSLC nano flow system, Dionex, UK) and CE-MS/MS were used. Resultant MS/MS data was analysed by Proteome Discoverer and compared against the Uniprot canine non-redundant database without enzyme specificity (Fig 9).

6.3.3 Statistical analyses

Statistical analyses were performed with JMP Pro 11 (SAS Institute, NC, USA), MedCalc (Medcalc Software, Belgium), and GraphPad Prism 7.00 (GraphPad Software, CA, USA). Study populations were described by medians and interquartile ranges. Non-parametric tests were used for comparisons of continuous variables between groups. Statistical significance was set at $p<0.05$. If multiple comparisons were made, corrections were applied, either by controlling familywise error rate (Bonferroni method, papers II and III) or, in paper IV, by controlling the false discovery rate (Benjamini & Hochberg, 1995).

Simple and multiple regression analyses (papers II and III) were used to evaluate associations between dependent variables of interest (cTnI, NT-proBNP, creatinine, SDMA and cystatin C concentrations) and different clinicopathological variables. Variables linearly associated with the dependent variable, and with a p-value of less than 0.2 in the univariate analyses, were included in the multiple regression modelling. The final model was constructed without interaction terms, in a backward stepwise manner. During this process, the variable with the highest p-value was removed in each step until only significant ($p<0.05$) variables remained. The resulting adjusted R^2 indicated the proportion of variability of the dependent variable that might be explained by the independent variables included in the final model. Multiple regression analyses were also used to construct models for mGFR-estimation using the three investigated biomarkers of GFR.

Partition analysis was performed in order to create a decision tree for clinical estimation of GFR using all available clinicopathologic variables of all dogs in paper III. Multiple regression, with mGFR as the dependent variable, and ROC-curve analysis was performed in order to evaluate added value of multiple markers for GFR estimation.

In paper IV, peptides differentially expressed between cases and controls in a training set were used to build classifying models with support vector machines (SVM), a software specifically designed to deal with large amounts of data. In SVM, each sample was regarded a "*p*-dimensional" vector where *p* represented the number of peptides in the sample. The SVM algorithm then constructed multidimensional separation planes between case and control vectors. The hyperplane with the largest distance to the nearest data-points on both sides

(=that best separated cases and controls) was selected. Sample classification in the validation process (healthy or CKD), was dependent of on which side of the hyperplane (case or control side), each vector (=validation set sample) was situated.

7 Main results

7.1 Kidney-related disease in Swedish dogs (paper I)

Incidence

The total number of dogs with a veterinary care insurance in Agria over the years 1995-2006 was 665,245. These dogs contributed with a total of 2,792,325 DYAR. The number of dogs with a veterinary claim of KD during the time they were insured was 4390. This corresponds to an overall IR for KD of 15.8 (CI: 15.3-16.2) cases/10,000 DYAR, approximately equally divided between female and male dogs. In most cases, the type (aetiology) of KD was not specified. The mean (±SD) and median (range) age at which the dogs were documented with the first episode of KD was 6.9 ± 3.3 and 7.4 (0-12) years, respectively.

In the breed-specific analysis (which, as previously mentioned, included only breeds contributing with > 10.000 DYAR to the Agria database), the Bernese mountain dog was the breed with the highest IR of KD (51 cases/DYAR). Swedish elkhound, Siberian husky and Finnish spitz were the three breeds with the lowest IR of KD (each five cases/DYAR) in this study (Fig. 10). In three breeds (flat-coated retriever, collie and golden retriever), female dogs had significantly higher IRs of KD than male dogs. There was no consistent change in yearly incidence rate of KD in this population of dogs insured over the years 1995-2006.

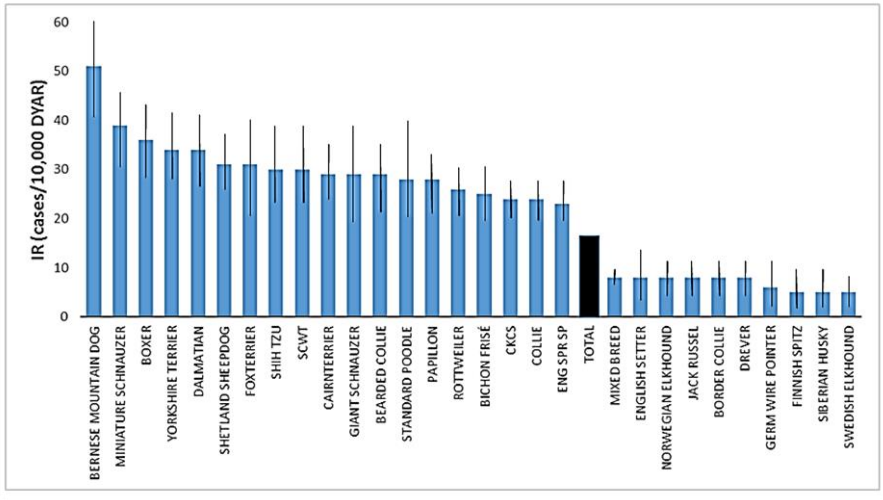


Fig 10. Incidence rates (cases/10,000 DYAR with 95% confidence intervals) of kidney-related disease in breeds with significantly higher or lower incidence rates than the average rate (15.8 cases/10,000 DYAR). DYAR; dog years at risk, SCWT; soft-coated wheaten terrier, CKCS; cavalier King Charles spaniel, Eng spr sp; English springer spaniel; Germ wire pointer, German wirehaired pointer.

Mortality

The total number of dogs with mortality insurance at Agria over the years 1995-2006 was 548.346. These dogs contributed with a total of 2.036.398 DYAR. Of these dogs, 1981 died because of KD, which corresponds to a total mortality rate of KD of 9.7 (CI: 9.3-10.2) deaths/10,000 DYAR. The total mortality rates for female and male dogs were 10.8 (CI: 10.2-11.5) and 8.6 (CI: 8.0-9.2) deaths/10,000 DYAR, respectively. Most commonly, the aetiology of KD leading to death of the dogs was not specified. The mean (±SD) and median (range) age at which the dogs died from KD was 6.6 ± 2.6 and 7.1 (0-10) years, respectively.

The Bernese mountain dog was the breed with the highest (49 cases/10,000 DYAR) and miniature dachshund the breed with the lowest (2 cases/10,000 DYAR) mortality rates of KD. In four breeds (Bernese mountain dog, flat-coated retriever, boxer and golden retriever), female dogs had significantly higher MRs of KD than male dogs.

7.2 Study population characteristics (papers II-IV)

The exact number of dogs included in the studies II-IV are shown in Fig 7. In total, 36 healthy dogs and 75 dogs with diagnosed or suspected CKD contributed with data to the studies. Of the dogs, 14 were mixed breeds, six were Labrador retrievers, six were golden retrievers, five were boxers and four or less were of 52 other breeds. The age of included dogs ranged between four months and 14.5 years.

7.3 Circulating biomarkers (papers II and III)

7.3.1 Biomarkers of cardiovascular homeostasis

N-terminal pro-B-type natriuretic peptide

In the univariate analyses, NT-proBNP increased with increasing plasma volume (PVf/kg), UPC, and creatinine concentration and with decreasing PCV, mGFR and albumin concentration. The two variables independently associated with NT-proBNP in this study were packed cell volume (PCV) and PVf/kg (R^2_{adj} = 0.42; p< 0.001). Concentrations of NT-proBNP in dogs in different stages of CKD and association between NT-proBNP concentration and mGFR are shown in Fig 11.

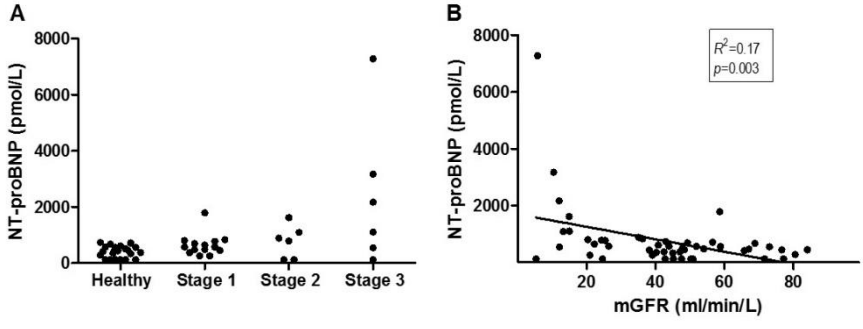


Fig 11. A. Concentrations of NT-proBNP in 23 healthy dogs and 27 dogs with CKD (stages 1-3). *B.* NT-proBNP increased with decreasing mGFR but this effect was not retained in the multiple regression analysis.

Cardiac Troponin I

In the univariate analyses, cTnI increased with increasing age, SBP, PVf/kg, BW and creatinine concentration. The three variables independently associated with cTnI in this study were age, SBP and BW (R^2_{adj} = 0.50; p< 0.001). Concentrations of cTnI in dogs in different stages of CKD and association between cTnI concentration and mGFR are shown in Fig 12.

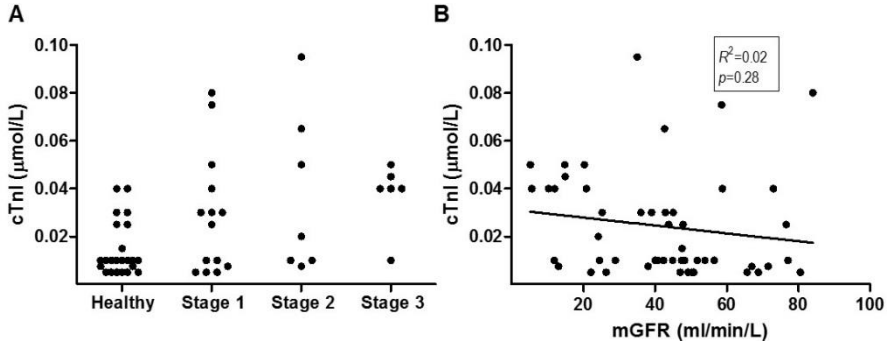


Fig 12. A. Concentrations of NT-proBNP in 23 healthy dogs and 27 dogs with CKD (stages 1-3). *B.* There was no association between cTnI and mGFR in 23 healthy dogs and 27 dogs with CKD.

7.3.2 Biomarkers of decreased GFR

Creatinine

The AUC of the creatinine concentration for detection of decreased mGFR (according to the pre-specified hospital mGFR cut-off <30.8 ml/min/L) was 0.98 (0.93-1.0) and the AUC for detection of mGFR below 37 ml/min/L was 0.94 (0.87-0.98). The "optimal" cut-off for detection of decreased GFR (<30.8 ml/min/L) in the dogs of this study was 127 µmol/L. Applying the pre-defined cut-off of 115 µmol/L, sensitivity and specificity of creatinine for diagnosis of decreased GFR (<30.8 ml/min/L) was 90% and 90%, respectively.

Ten dogs were falsely categorised with regard to mGFR status, using creatinine concentration with the pre-defined cut-off value. In Table 1A, SDMA and cystatin C concentrations in the 10 dogs falsely classified by creatinine, are shown. Measured GFR, BW, PCV, and phosphate concentration were the variables independently associated with creatinine concentration (R^2_{adj}=0.74; P<0.0001).

Symmetric dimethyl-arginine

The AUC of SDMA for detection of decreased GFR (according to the pre-specified hospital mGFR cut-off <30.8 ml/min/L) was 0.96 (0.91-0.99) and the AUC for detection of a mGFR below 37 ml/min/L was 0.94 (0.87-0.98). The "optimal" cut-off for detection of decreased GFR in the dogs of this study was 16 µg/dL. Applying the pre-defined cut-off of 14, sensitivity and specificity of SDMA for diagnosis of decreased GFR (<30.8 ml/min/L) was 90% and 87%, respectively.

Twelve dogs were falsely categorised with regard to mGFR status using SDMA concentration with the pre-defined cut-off value. In Table 1B, the creatinine and cystatin C concentrations in the 12 dogs falsely classified by SDMA, are shown.

There was a difference between concentrations of SDMA in healthy dogs, and dogs in IRIS stages 1-3 of CKD (except between stage 2 and 3). Stage 4 dogs were too few to be included in the statistical comparison of biomarker concentrations between groups. Measured GFR, phosphate concentration, albumin concentration and USG were the variables independently associated with SDMA concentration (R^2_{adj}=0.71; P<0.0001).

Cystatin C

The AUC of cystatin C for detection of decreased GFR (according to the pre-specified hospital mGFR cut-off <30.8 ml/min/L) was 0.87 (0.79-0.93) and the AUC for detection of mGFR below 37 ml/min/L was 0.83 (0.74-0.90). The "optimal" cut-off for detection of decreased GFR in the dogs of this study was 0.47 mg/L. Applying the "defined" cut-off of 0.49 mg/L, corresponding to the same sensitivity as creatinine (90%), specificity of cystatin C concentration for diagnosis of decreased mGFR (<30.8 ml/min/L) was 75%. There was a difference between concentrations of cystatin C in healthy dogs, and dogs in IRIS stages 1-3 of CKD (except between stage 1 and 2). Measured GFR, age, PCV, calcium concentration and USG were the variables independently associated with cystatin C concentration (R^2_{adj}=0.62; P<0.0001).

Multivariable analyses for estimation of mGFR

The combination of creatinine and SDMA concentrations for detection of decreased mGFR in a multiple regression model resulted in an adjusted R^2 of 0.67 and an AUC of 0.98. For the combination of creatinine and cystatin C concentrations, adjusted R^2 was 0.65 and the AUC was 0.99. When creatinine, SDMA and cystatin C concentrations were all used to construct a model, adjusted R^2 was 0.68 and AUC was 0.99. In the partition model (Fig 13) for

diagnosis of decreased mGFR, the resulting decision tree contained only creatinine and cystatin C. At one location in the decision tree, cystatin C was interchangeable with BW (red boxes).

Table 1. *Results of other markers in dogs falsely categorised (by creatinine and SDMA) dogs*

A) Dogs misclassified by creatinine	mGFR ml/min/L	Creatinine µmol/L	SDMA µg/dL	Cystatin C mg/L
Breed	(30.8)	(115)	(14)	(0.51)
Greyhound	43	136	13	0.49
Greyhound	43	125	13	0.23
Labrador retriever	41	117	11	0.43
Large Swedish hunting breed	49	121	14	0.98
Bernese mountain dog	43	117	16	0.62
German shepherd	33	116	14	0.36
Cane corso	36	120	14	0.41
Bedlington terrier	↓ 29	105	16	0.51
Small mixed breed	↓ 27	104	12	0.58
Tibetan terrier	↓ 25	100	14	1.54
B) Dogs misclassified by SDMA	mGFR ml/min/L	SDMA µg/dL (14)	Creatinine µmol/L	Cystatin C mg/L
Breed	(30.8)		(115)	(0.51)
Golden retriever	44	15	92	0.53
Golden retriever	73	15	76	0.23
Mops	38	15	110	0.73
Kerry blue terrier	47	15	90	0.39
French bulldog	47	16	61	0.32
Soft-coated wheaten terrier	48	15	81	0.89
Boxer	38	19	78	0.38
Bernese mountain dog	43	16	117	0.62
Large mixed breed	47	15	73	0.44
Basenji	↓ 29	13	127	0.50
Small mixed breed	↓ 27	12	104	0.58
Tibetan terrier	↓ 25	14	100	1.54

A) Breed, measured GFR (mGFR) and creatinine, SDMA and cystatin C concentrations in the 10 dogs falsely categorised by creatinine with regard to mGFR status (cut-off 30.8 ml/min/L). ***B)*** Breed, mGFR and SDMA, creatinine and cystatin C concentrations in the 12 dogs falsely categorised by SDMA with regard to mGFR status. Green-coloured fields indicate a "correct" result and red fields indicate an "incorrect" result, with regard to mGFR status.

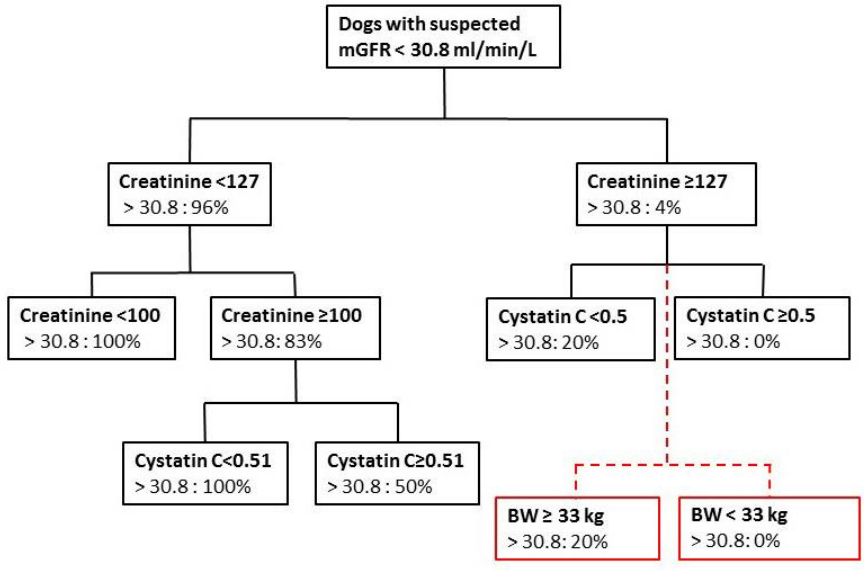


Fig 13. Resultant decision tree in partition modelling for estimation of mGFR-status in clinically stable dogs. Percentages represent proportion of dogs in each box with mGFR >30.8 ml/min/L, ("normal"). Creatinine, SDMA and cystatin C concentrations were all included as independent variables, but SDMA concentration was not used in the final algorithm. The resultant decision tree was identical when all available clinical variables were included, but bodyweight was interchangeable with cystatin C concentration for partitioning dogs with a creatinine concentration ≥127 µmol/L (red boxes).

7.4 Urinary CE-MS analysis (paper IV)

In total, 5398 different peptides were identified in all samples (Fig 14). One hundred and thirty-three peptides were differentially excreted between healthy dogs and those with CKD (Fig 15). Of these 133 peptides, 35 were sequenced, and most (n=32) were fragments of collagen I (α1 or α3 chains), one was a fragment of collagen IV and two were fragments of uromodulin. Two peptide models were developed, one (133P) containing all 133 differentially expressed peptides, and one (35P) containing all 35 differentially expressed peptides that were sequenced in the present study. Both models predicted disease status (healthy vs CKD) of the dogs in the independent validation cohort with an AUC of 0.88 (95% CI: 0.72-1.0). Two out of the three inconclusive dogs were characterised as healthy and one was characterised as CKD.

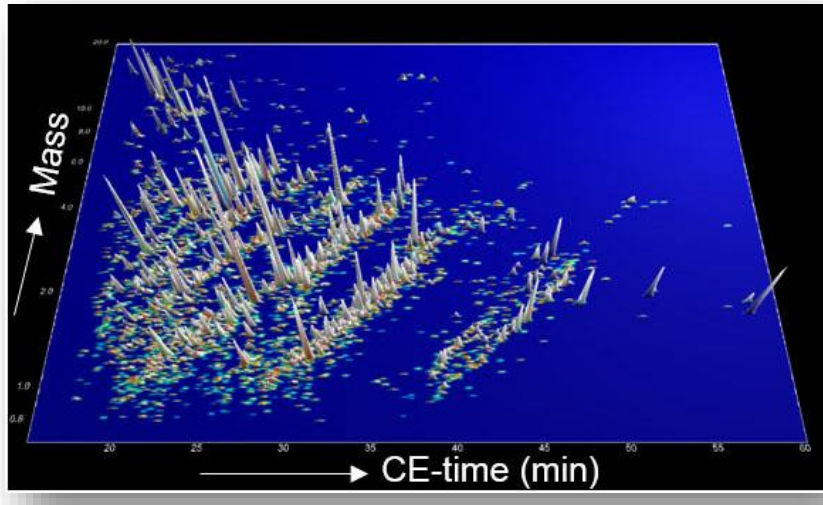


Fig 14. All 5398 identified peptides in urine samples from 50 dogs, with and without CKD, in paper IV. Peptide molecular mass is three-dimensionally plotted against normalised capillary electrophoresis migration time.

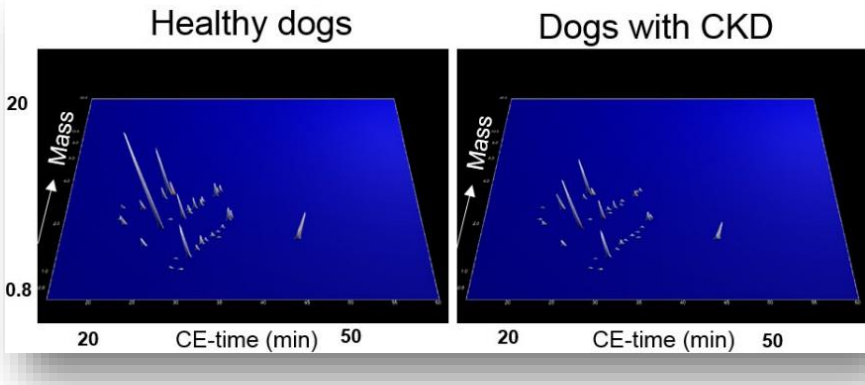


Fig 15. Differentially expressed peptides (n=133) in healthy dogs and dogs with chronic kidney disease, in paper IV. Peptide molecular mass is three-dimensionally plotted against normalised capillary electrophoresis migration time.

8 General discussion

8.1 Epidemiology of CKD

The overall IR of KD in this large dog population was 15.8 cases/10,000 DYAR. Incidence rates of a few other diseases have been calculated in similar populations of dogs in the Agria database: The IR of KD was similar to that of atopic dermatitis (17 cases/10,000 DYAR) and approximately half of the IR of intervertebral degenerative disc disease (27.8 cases/10,000 DYAR) (Bergknut *et al.*, 2012; Nodtvedt *et al.*, 2006). The IR of KD was however less frequent than dystocia (57 cases/10,000 DYAR) and pyometra (199 cases/10,000 DYAR) (Jitpean *et al.*, 2012; Bergstrom *et al.*, 2006), which were both calculated using only female dogs at risk. The mortality rate of KD was 9.7 deaths per 10.000 DYAR, which was similar to the MR of intervertebral disc disease (9.4 deaths/10,000 DYAR) and about half of the MR of heart disease (21.4 deaths/10,000 DYAR) in previous studies on dogs in the Agria database (Bergknut *et al.*, 2012; Egenvall *et al.*, 2006).

Animal health insurance databases, unlike hospital data, contain information about the whole population at risk (which is necessary in order to investigate incidence rates of disease) and not only information regarding clinical events. However, interpretation of IRs beyond comparison of incidence between different diseases may be difficult. In paper I, an alternative interpretation was discussed, in that 10,000 DYAR might represent 1000 dogs (each living 10 years). Of these, approximately 16 dogs developed KD, which equals a cumulative proportion, or period prevalence, of 1.6 %.

All epidemiologic measurements of KD frequency are likely to represent underestimations of the truth because of the insensitivity of current diagnostic methods. However, this probably affects prevalence measurements more than incidence rates. Misdiagnosis is another possible source of error in insurance

database analysis, which may result in both under- and overestimation of disease occurrance. Because of limited data resolution, the occurrence of misdiagnosis cannot be controlled. Validity of secondary databases may vary, but it can be high, depending on how similar the purpose of data collection was to the research question at hand (Egenvall *et al.*, 2009). A validation of data against veterinary practice records has been performed, and agreement between information in practice records and in the Agria database was excellent for information regarding breed and sex and fair for information regarding date of birth and for specific diagnoses (Egenvall *et al.*, 1998). Possibly, choice of specific insurance companies by owners of some breeds, may also influence results.

In the breed-specific analysis of paper I, it was shown that a number of dog breeds had a higher, and some breeds a lower, incidence rate of KD than the population as a whole. The IR of KD in the Bernese mountain dog, 51 cases/10,000 DYAR, was over three times as high as the IR of all dogs (15.8 cases/10,000 DYAR). Shetland sheepdog, one of the breeds in which KD has previously not been recognised as frequent, was shown in paper I to have a high IR and MR of KD. For Shetland sheepdog, the IR was 31 cases/10,000 DYAR, which was twice as high as the average of all breeds. The MR of KD for this breed was the second highest of all included breeds, >2.5 times the average MR. This type of information may help in diagnostic decision making when assigning different probabilities to relevant differential diagnoses for an individual under investigation. Importantly, in order not to introduce bias, many breeds were not included in this comparison because the number of DYAR was considered too low to allow meaningful data.

8.2 Cardiovascular-renal interaction

In paper II, it was shown that neither NT-proBNP nor cTnI concentration were independently associated with GFR. As GFR decreases in CKD, circulating volume status and myocardial cell integrity may both be affected. In this situation, circulating concentration of NT-proBNP and cTnI are expected to change accordingly. If, however, passive accumulation of these biomarkers occurs, the diagnostic value for detection of cardiovascular pathology is lessened. According to the results of paper II, neither of these markers passively accumulate as GFR decreases in dogs with stable CKD. The situation may however be different for dogs with acute or acute-on-chronic kidney disease.

Circulating volume status

In paper II, circulating volume (PVf/kg) was shown to increase with decreasing mGFR. This might explain an increase in NT-proBNP concentration with decreasing mGFR in dogs without cardiac pathology (Fig 11 B), because NT-proBNP is released in response to cardiac stretch, irrespective of underlying reason. Changes in circulating volume secondary to CKD is one example and might possibly explain increased concentrations of NT-proBNP in at least some of the dogs with renal compromise described in previous studies (Raffan *et al.*, 2009; Schmidt *et al.*, 2009; Boswood *et al.*, 2008).

In addition to the effect of different disease states on concentrations of NT-proBNP, this marker has also been shown to differ considerably between breeds and to have a comparably high individuality (Ruaux *et al.*, 2015; Sjostrand *et al.*, 2014). Consequently, interpretation of a single NT-proBNP result from an individual dog in a clinical situation in relation to suggested, population-based, clinical cut-offs should be done with caution. As for all tests, knowledge of disease pathophysiology and performance characteristics of the test are important for optimal interpretation of test results.

Myocardial cell integrity

In paper II, cTnI concentration was higher in the dogs with CKD (as a group), than in healthy dogs. There was, however, no association between cTnI and mGFR (Fig 12 B). Subclinical myocardial cell damage is a plausible reason for the (comparably small) increases in circulating concentrations of cTnI observed in some of the dogs in this study. As discussed in section 3.5, subclinical myocardial damage of unknown aetiology occurs in people with CKD as well.

Earlier observations (Silvestrini *et al.*, 2012; Ljungvall *et al.*, 2010; Serra *et al.*, 2010) of an age-dependence of cTnI concentration in dogs were confirmed in the multiple regression analysis in paper II. However, the absolute increase of cTnI with age in the healthy population was comparably small (lower than the limit of detection for many cTnI assays used in the clinic), and therefore probably negligible when this analysis is used clinically to assess myocardial cell integrity.

8.3 Diagnosis of CKD

8.3.1 GFR assessment

Overall diagnostic value of SDMA and cystatin C

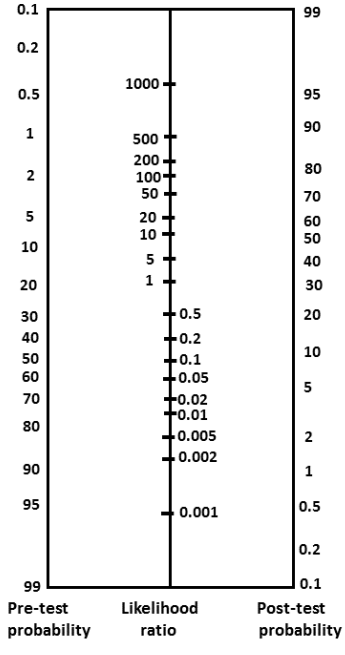
In paper III, ROC-curve analysis was used to investigate the overall diagnostic value of SDMA and cystatin C concentrations as markers of decreased GFR. The overall value of SDMA was similar to that of creatinine. The value of cystatin C as a marker of mGFR was, however, inferior to both creatinine and SDMA. These results may appear disappointing, but most (or all) surrogate markers of GFR are expected to perform approximately as creatinine does. The association between concentration of circulating biomarkers and GFR is expected to be non-linear and best described by an exponential function.

Clinical cut-offs for creatinine, SDMA and cystatin C

Results of ROC-curve analyses, although recommended for comparison between different tests, have little direct relevance for interpretation of results in the clinical use of biomarkers. In order to calculate properties of biomarker tests, such as sensitivity and specificity, cut-off values are used. In paper III, cut-offs to indicate decreased GFR for creatinine and SDMA were obtained from the laboratories where analyses were performed, the University Animal Hospital in Uppsala for creatinine (115 µmol/L), and Idexx Laboratories in Germany for SDMA (14 mg/dl). The sensitivity of creatinine and SDMA was exactly the same (90%) when applying these cut-offs, and the specificities very similar (90% versus 87%), in accordance with the ROC-curve analysis. A "defined" cut-off for cystatin C, which corresponded to an identical sensitivity as that of creatinine (90%), was chosen. The specificity of cystatin C at the defined cut-off was 75%, again showing the inferior diagnostic value of cystatin C compared to the other two markers.

The cut-offs that corresponded to the "best combination" of sensitivity and specificity, identified in the ROC-curve analysis, were slightly higher than the pre-defined cut-offs for both creatinine and SDMA (126 µmol/L and 16 mg/dl, respectively). By use of the pre-defined, currently clinically used, cut-offs for decreased GFR, high sensitivities of the tests are prioritized and consequently, specificity is compromised. Cut-offs used to indicate decreased GFR may be adjusted depending on desired biomarker performance, keeping in mind that sensitivity and specificity depend on each other.

Test sensitivity and specificity, although clinically useful in some circumstances, are sometimes confused with positive predictive value and post-test probability, and this may result in diagnostic error. In paper III, interval LRs for creatinine, SDMA and cystatin C concentrations are suggested. These ratios are used to modify pre-test probability of disease. In a clinical setting, pre-test probability refers to the individual dog, taking into account the history, physical examination and results of any previous test results. Assessment of pre-test probability is important both to the very decision of whether to perform a diagnostic test, and to the interpretation of test results. The Fagan nomogram (Fig. 16) for Bayes´s theorem may be used to quickly estimate the change of disease probability using the LRs of the biomarkers investigated in paper III (Fagan, 1975). A straight line is drawn from the pre-test probability through the LR, to indicate the post-test probability on the right side of the graph. Evident when using the nomogram is that post-test probability, although not immediately obvious or intuitive, depends heavily on pre-test probability. Because a precise estimation of pre-test probability is difficult, the nomogram may be used qualitatively (Medow & Lucey, 2011). Both pre- and post-test probability may then be thought of in terms of very unlikely, unlikely, uncertain, likely or very likely.



The term “clinical accuracy” has been proposed to indicate the ability of a test to modulate probability of disease (Fanshawe *et al.*, 2018). At least for some ranges of results of all three biomarkers investigated in paper III, the clinical accuracy is very high, as reflected by high interval LRs. The exact cut-offs for the different intervals of LR of the three biomarkers may, however, differ between assays.

Fig 16. Fagan nomogram, used to estimate post-test disease probability when the likelihood ratio of a test is known (Fagan T.J. 1975). Likelihood ratios are presented for SDMA, cystatin C and creatinine concentrations in paper III.

Creatinine

Results of laboratory tests may vary with certain signalment features of the dog under investigation for CKD. As mentioned above, serum creatinine concentration is dependent on muscle mass, which in turn differs between dogs of different breeds and sizes. Results of paper III support this, as several large breed dogs were falsely categorised by creatinine (azotaemia despite mGFR below the cut-off value). Also, in three small breed dogs, creatinine concentrations were below the cut-off for azotaemia despite a low mGFR (Table 1A). If a stable creatinine concentration (=at steady state), regardless of the reference interval, does not `match` the muscle mass or breed size of a dog with suspected kidney disease, additional tests of GFR may be indicated. This can be accomplished by GFR measurement by any available method, or by use of other indirect biomarkers of GFR, such as SDMA or cystatin C.

SDMA

In paper III, the overall diagnostic value of SDMA was similar to that of creatinine. Despite this, SDMA may be of clinical use in selected dogs. However, the claim that the concentration of SDMA may be used to detect decreased GFR at an earlier point in time of disease development is inaccurate, as this depends entirely on where the cut-off is placed. This is similar to the situation for most, if not all, quantitative biomarkers including creatinine.

An example of how SDMA concentration may be used in staging of dogs with CKD is provided on the website of IRIS (IRIS, 2016). It is suggested that in dogs of IRIS stage 2 with an SDMA concentration persistently above 25 µg/dl, GFR may be underestimated by the creatinine concentration. Our results are in agreement with this statement. There was a comparably large overlap in SDMA concentrations between stage 2 and stage 3 dogs, and in the 12 dogs in IRIS stage 2, SDMA more accurately than creatinine reflected mGFR (R^2 = 0.47 vs 0.19 for creatinine). This was not true for the 12 dogs in stage 3, however. In these dogs, creatinine reflected mGFR more accurately than SDMA did (R^2 = 0.60 vs 0.20 for SDMA). Further investigations are needed to confirm these differing associations of creatinine and SDMA concentration with GFR in other populations of dogs in stage 2 and 3. For dogs in stage 1, as well as for healthy and inconclusive dogs, neither creatinine nor SDMA concentrations were strongly associated with mGFR. This has previously been shown for creatinine (van den Brom & Biewenga, 1981).

As an adjunct test to creatinine, interpreted with the pre-defined cut-off or with the interval LRs proposed in paper III, this assay is probably of clinical use as a biomarker of GFR in addition to creatinine. However, the proposed interval

LRs for SDMA concentration in paper III should be further evaluated in other patient populations.

Cystatin C

Overall diagnostic performance of cystatin C concentration as a marker of decreased mGFR was inferior to both creatinine and SDMA according to paper III. The reason for the inferior diagnostic performance of cystatin C is unknown. The molecular weight (MW) of cystatin C (13.4 kDa) is substantially higher than those of creatinine (0.11 kDa), SDMA (0.20 kDa) and the radiopharmaceutical used for scintigraphy (^{99}Tc= 0.99 kDa; DTPA=0.39 kDa). Therefore, differing sieving coefficients of these molecules (cystatin C vs creatinine, SDMA and ^{99}Tc-DTPA) may indicate pathology at the level of the glomeruli (Grubb *et al.*, 2015). In human medicine, a syndrome referred to as ´shrunken pore syndrome´ has recently been identified. This syndrome is suspected when eGFR calculated with cystatin C-based equations is ≤ 60% of the eGFR calculated with creatinine-based equations at the same point in time (Grubb *et al.*, 2015). Shrunken pore syndrome is thought to occur because of shrinkage of pores in the glomerular filtration barrier, resulting in decreased filtration of molecules with MWs of between approximately 6.5-70 kDa, which are normally relatively freely filtered (high sieving coefficients) at the glomerulus. Whether the syndrome occurs in dogs, and thus might have influenced results of this study, is unknown.

Individuality is important for diagnostic utility of a test when a population-based cut-off is used, as in paper III (Petersen *et al.*, 1999). The low individuality of cystatin C in comparison with that of creatinine has been considered an advantage of cystatin C when used as a marker of GFR in people (Keevil *et al.*, 1998). Later studies have, however, reported a similar individuality of creatinine and cystatin C in people (Carter *et al.*, 2016; Andersen *et al.*, 2010b; Bandaranayake *et al.*, 2007). In dogs, individuality of creatinine and cystatin C has been shown to be similar in two studies (Pagitz *et al.*, 2007; Jensen & Aaes, 1993). This needs further investigation because individuality is an important property that influences diagnostic value of markers, when evaluated using a population-based cut-off value.

Age was independently associated with cystatin C concentration in paper III. In two of the 30 healthy dogs, cystatin C concentration was > 0.5 mg/L (0.55 and 0.77). These dogs were 12 and 14 years old, respectively. Higher concentrations of cystatin C in older dogs without disease of the kidneys have been shown in some studies (Monti *et al.*, 2012; Braun *et al.*, 2002), but in others no effect of age was seen (Miyagawa *et al.*, 2009; Wehner *et al.*, 2008). Evaluations of a potential age-dependence of cystatin C as well as of other markers of GFR are complicated by the possibility of age-related subclinical

decline of GFR in healthy individuals. In this study the increase in cystatin C concentration with age was independent of mGFR and therefore, age might be taken into consideration if this PETIA assay with a clinical cut-off value of 0.5 mg/L is used (as in the partition model, Fig 13). If the higher clinical cut-off of 0.9 mg/L is used (as in the proposed interval LRs in paper III) however, the effect of age can probably be ignored. The cystatin C-concentration was above 0.9 mg/L in only one of all dogs with mGFR below the cut-off value (n=68).

Advantages with the cystatin C-assay used in paper III are the commercial availability of the reagent and the fact that it can be analysed in biochemistry instruments that are already in use at many clinical laboratories. As an adjunct test to creatinine concentration, interpreted by the partition analysis (decision tree) or the interval LRs proposed in paper III, this assay might be of clinical use in selected patients. However, all proposed clinical cut-offs for cystatin C concentration in paper III should be further evaluated in other patient populations. As noted above, age of the dog under investigation should be taken into consideration in the interpretation of cystatin C concentrations, especially when using the decision tree.

Value of SDMA and cystatin C as additional markers of GFR

As shown in Table 1A, SDMA (or cystatin C) analysis in addition to creatinine concentration may increase diagnostic yield for individuals that are falsely categorised by creatinine. Many of these dogs were either large or tiny, with large or small muscle mass, respectively. No unifying patient characteristics could be identified that could aid in identifying dogs that may be falsely categorised using the SDMA-test, however (Table 1B). In this regard, the dependence on muscle mass (that is usually considered a drawback for creatinine) may be considered a slight advantage, in that it is known when to doubt a particular creatinine result, but not when to doubt an SDMA-result. This is clinically relevant, as the SDMA test falsely categorised (with regard to mGFR) approximately the same number of dogs as did the creatinine test. Both markers are valuable, but a reasonable recommendation could be to depend on creatinine as the primary test of GFR and to interpret SDMA concentration in light of creatinine concentration.

When Table 1 (falsely categorised dogs) was constructed, mGFR for each dog was known. This is usually not the case in a clinical situation. It would be interesting to prospectively investigate the value of adding SDMA and cystatin C concentrations to the clinical investigation of dogs for which the creatinine concentration is suspected to be a less reliable indicator of GFR, as in the evaluation of a large dog with mild azotaemia, or a small or emaciated dog with a creatinine concentration close to the cut-off value for decreased GFR.

Value of multiple clinicopathological variables for GFR assessment

In diagnostic accuracy studies, the value of tests are usually investigated as if the test-result had to stand on its own. This is seldom the case in a clinical situation. The construction of diagnostic models including other available clinical information in the evaluation of diagnostic tests has been encouraged in order to improve diagnostic research (Moons *et al.*, 1999). In paper III, available clinicopathological variables from all dogs were included in the partition model for diagnosis of decreased mGFR. Only creatinine and cystatin C appeared in the resultant decision tree. The interpretation of this is that, *overall*, SDMA concentration does not offer much new information when creatinine concentration is already known. In accordance with this, creatinine concentration (with different cut-offs) was the only variable left in the decision tree if only SDMA and creatinine were entered into the model (data not shown). However, as discussed above, for some dogs, SDMA is probably highly valuable as an adjunct test.

The three GFR biomarkers (creatinine, SDMA and cystatin C) were also entered into multiple regression models with mGFR as the dependent variable. Addition of SDMA to creatinine only marginally improved model performance (adjusted R^2 of 0.67 for creatinine and SDMA together, compared to the R^2 for either marker alone; 0.62). The same was true when instead adding cystatin C (R^2 = 0.46) together with creatinine (R^2_{adj}=0.65), and when including all three biomarkers as variables in the model (R^2_{adj}=0.68). The resultant AUCs of different combinations of biomarkers in ROC-curve analyses were also very similar to each other, and to the AUC of creatinine alone (0.98). This supports the results in the initial ROC-curve analysis; that overall diagnostic value of creatinine and SDMA is similar. This may be interpreted as supportive of the main finding of paper III, that the overall value of adding SDMA or cystatin C to creatinine is rather low. The proposed value of marker combinations for selected individuals, however, might possibly explain the slight improvements in adjusted R^2 for combinations of markers compared to the R^2 for each individual marker alone.

Association of age and bodyweight with glomerular filtration rate

There was no association between age and mGFR, or between BW and mGFR, in the 30 healthy dogs (or in all dogs with mGFR > 30.8 ml/min/L, n=68) included in paper III. On average, small breed dogs have higher GFR than large breeds do (Miyagawa *et al.*, 2010; Bexfield *et al.*, 2008). The fact that only four of the 68 dogs with mGFR above the cut-off value in paper III weighed >35 kg

might explain the lack of a negative association between BW and GFR in paper III.

An age-related decrease in GFR in small dogs (<12.4 and <15.1 kg, respectively) has previously been shown (Miyagawa *et al.*, 2010; Bexfield *et al.*, 2008). Among included dogs in paper III, no association was evident between age and mGFR in healthy dogs, or in the 68 dogs with mGFR > 30.8 ml/min/L. Neither was there an association between age and mGFR when analysing dogs <15 kg (with mGFR > 30.8 ml/min/L, n=19) separately.

8.3.2 Urinary capillary electrophoresis and mass spectrometry

In paper IV, 133 naturally occurring peptides that were differentially present in urine from dogs with and without CKD were identified. The classifying model, 133P, constructed and validated in a separate cohort of dogs, could separate dogs with CKD from healthy dogs with a sensitivity of 80% and a specificity of 80%. These results may be compared to those of the human counterpart of the 133P-model (CKD273) when applied to the first validation group of people (Good *et al.*, 2010). In that study, the sensitivity of the CKD273-model was 85% (95% CI: 75-91) and the specificity 100% (95% CI: 91-100). In total, 230 patients with CKD and 379 "seemingly healthy" people were used to create the CKD273-model. With increasing data from dogs, classification may further improve, because the number of applicable polypeptides depend on the number of cases in the model construction cohort (Weissinger *et al.*, 2004). On the other hand, the control dogs used in paper IV were all very well characterised, in contrast to the controls in the corresponding human study, and that may have reduced the risk of "noise" that might have been introduced by possibly including control individuals with subclinical disease.

One possible reason for false positive and negative results in a diagnostic accuracy study, such as paper IV, is that the new test under investigation (CE-MS) actually performs better than the reference test (clinical evaluation using serum creatinine concentration, scintigraphy, urinalysis and renal US). Also, CKD aetiology and progression mechanisms may be of importance for results. One of the two dogs falsely characterised by the 133P-model as healthy was diagnosed with polycystic kidney disease (PKD). This is a disease in which multiple cysts grow and gradually replace the renal parenchyma. It is possible that the pathophysiology of PKD, on a molecular level, differs from that of many other aetiologies of CKD. In the study in which the CKD273-model was constructed, PKD was not mentioned among the different CKD aetiologies of included people (Good *et al.*, 2010).

Urine from three inconclusive dogs were analysed by CE-MS analysis in paper IV. Two of these dogs, included because of abnormal renal architecture detected by ultrasound, were classified as "healthy" by the 133P-model, and neither dog had evidence of reduced renal function on follow-up examinations (creatinine concentration only) at least two years after study inclusion. The third dog was included six months after treatment of pyelonephritis. At the time of inclusion, this dog was non-azotaemic, non-proteinuric and the total GFR was > 30.8 ml/min/L. Renal ultrasound was unremarkable. Therefore, this dog was not included in the "CKD-group". However, the mGFR of the left kidney, 15 ml/min/L, was half of that of the right kidney, 34 ml/min/L (which would not have been appreciated using only routine diagnostics), and the 133P-model categorized this dog as CKD. At the follow-up visit two and a half years after inclusion, the dog was considered healthy by the owners. The mGFR of the left kidney was similar to the first measurement (14 ml/min/L), but mGFR of the right kidney was at this point similar to that of the left kidney (19 ml/min/L). Total mGFR was, however, still above the cut-off at 30.8 ml/min/L and serum creatinine concentration was not increased compared to the concentration at study inclusion. Our interpretation is that this dog might have had slowly progressive CKD at inclusion, as reflected by the 133P-model, but that was not detectable with routine diagnostic methods. Further clinical follow-up is planned for all three dogs.

Differences in urine concentration of samples (pre-renal influences) was controlled for by normalisation of peptides to internal standard peptides when peptide abundance was assessed in paper IV. Normalisation may be performed in different ways in urine. One study using CE-MS compared normalisation to urinary creatinine concentration or exogenous stable isotope-labelled peptide standards (absolute quantification), with normalisation to highly abundant collagen fragments, "internal peptides", by ion counting (relative quantification). The results indicated that relative identification using internal peptides was a reasonable alternative. Adding exogenous isotope-labelled peptides did not appear to confer any additional benefit (Jantos-Siwy *et al.*, 2009).

The addition of a urinary proteomic-based model for detection of CKD could be useful in a clinical situation as an adjunct to the routine diagnostic methods currently available (Fig 17). This is especially true for tubulointerstitial disease without proteinuria, which is usually limited to comparably late stages of disease.

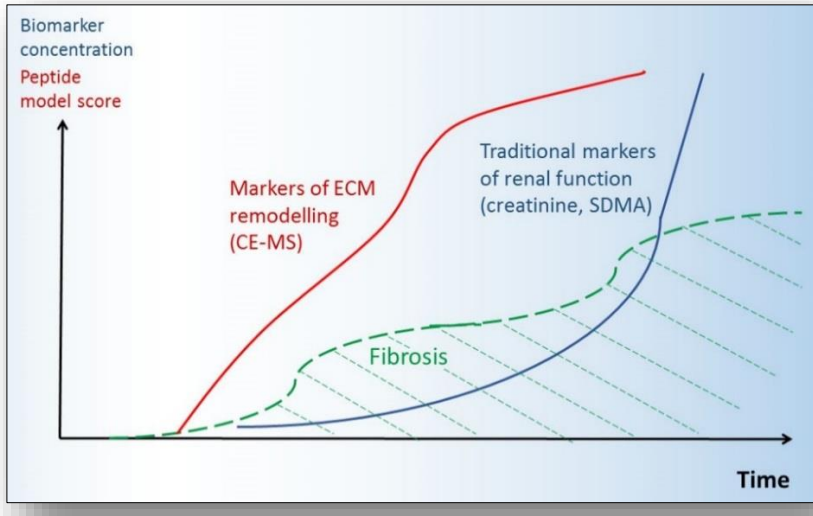


Fig 17. Detection of molecular processes related to fibrosis may provide a future diagnostic option for early diagnosis of canine CKD, compared to the markers of glomerular filtration rate used today. SDMA; symmetric dimethyl arginine, ECM; extracellular matrix, CE-MS; capillary electrophoresis and mass spectrometry.

The urinary peptidome in intrarenal fibrosis

As mentioned in section 4.3.2, peptides identified in the CE-MS process are naturally occurring in the urine. No proteolytic step (that produces "artificial" peptides) is needed during sample preparation (Mischak *et al.*, 2013). Detection of changes in the urinary peptidome that may be linked to CKD not only provides an exciting new alternative way of early diagnosis to explore further, but may also shed some light on pathophysiological mechanisms contributing to renal damage. In paper IV, 30 out of the 133 differentially expressed peptides used to create the 133P-model were sequenced. Most peptides (n=32) were fragments of collagen I, one peptide originated from collagen IV and two were uromodulin (previously called Tamm-Horsfall mucoprotein) fragments. All peptides were present in lesser amounts in dogs with CKD than in healthy dogs.

Fragments of collagen are abundant in the human urinary peptidome as well, and probably reflect physiological turnover of the ECM (Coon *et al.*, 2008). The renal ECM is composed of collagen type I, III, V, VI and XV, polysaccharides, glycoproteins and glycosaminoglycans (Bosman & Stamenkovic, 2003). In the process of renal fibrosis, excessive intrarenal accumulation of these components occurs. Collagen I and III have been shown to accumulate early after an insult

to the kidneys in people and rats (Johnson *et al.*, 2002; Sharma *et al.*, 1993). Among the peptides differentially expressed between people with CKD and healthy controls, fragments of collagen type I are present in lower amounts in CKD, similar to the result in the dogs in paper IV (Roscioni *et al.*, 2013; Alkhalaf *et al.*, 2010; Good *et al.*, 2010). This decrease in collagen fragment abundance is thought to reflect attenuation of extra-cellular matrix degradation by proteases, resulting in intrarenal fibrosis that constitutes the common pathway in progressive CKD of different aetiologies (Rossing *et al.*, 2008; Eddy & Neilson, 2006). Collagen fragments have been shown to represent valuable peptidomic biomarkers for early CKD detection in people (when healthy individuals and those with CKD are to be distinguished), but less valuable in peptide classifying models constructed with the aim to differentiate different aetiologies of kidney diseases from each other (Siwy *et al.*, 2016).

Fragments of uromodulin, which is found in renal tubules of people and dogs, were less abundant in the urine of dogs with CKD than in the healthy ones in paper IV. Intact uromodulin has been shown to be less abundant in urine of people with CKD than in healthy ones (Chakraborty *et al.*, 2004). Recent studies have proposed a role of uromodulin in the development of intrarenal ECM deposition by early tubular back-leakage, leading to abnormal interstitial uromodulin deposition inducing inflammation, subsequent fibrosis and progression of CKD (Prajczer *et al.*, 2010). A possible link between this role of uromodulin in fibrosis and the decrease in uromodulin fragments in CKD dogs in paper IV needs further investigation.

The human peptidome model CKD273, although initially created for early diagnosis of CKD, was subsequently shown to be strongly associated with CKD progression, further strengthening the hypothesis that the differentially expressed peptide fragments reflect an ongoing intrarenal fibrosis process (Schanstra *et al.*, 2015; Gu *et al.*, 2014; Argiles *et al.*, 2013; Roscioni *et al.*, 2013). A potential association between the 133P-model and progression was not possible to assess in paper IV because of its cross-sectional design. Although there was a time aspect to the diagnosis of CKD ($\geq$ three months), it was not known for every included dog if they had progressive or non-progressive disease. Included dogs are followed up in order to assess this aspect at a later date.

A possible detection of ongoing intrarenal fibrosis by the 133P-model is intriguing, because if this model is further validated and found useful, the peptides that comprise this classifying model may be perceived as "active kidney injury biomarkers". Such markers detect the recurrent (or sustained) injury suggested to characterise progressive CKD. A call for investigation of active kidney injury markers in veterinary medicine was recently made (Cowgill *et al.*,

2016). These authors also speculate that a panel of biomarkers probably will be needed to detect both active insult and recovery processes. Similarly, because of the substantial heterogeneity of CKD, the application of diagnostic panels containing multiple biomarkers has been advocated for urinary proteomics (Klein *et al.*, 2016; Fliser *et al.*, 2007). Specifically in CE-MS analysis, it was shown that classifying models that contained fewer peptides consistently underperformed compared to models with larger numbers of peptides, probably as a result of overfitting (Mischak *et al.*, 2013). Therefore, the 133P-model (containing 133 peptides) may have more potential than the 35P model (containing only the 35 sequenced peptides) that was also constructed in paper IV.

The potential for early diagnosis of CKD using a marker that probably detects ongoing fibrosis depends on when in the process of CKD the fibrotic process starts. The ambition of this project was to include dogs primarily in the early stages of CKD, in order to investigate early diagnosis. Consequently, many dogs with CKD were in stage 1 or 2 on the day of inclusion. Thus, the 133P-model was constructed using individuals with comparably early disease (at least considering the diagnostic options in clinical use today). Data from clinical studies in people with CKD also argue for a significant involvement of fibrosis in mild or subclinical stages of CKD (Argiles *et al.*, 2013; Prajczer *et al.*, 2010; Rossing *et al.*, 2008). Additionally, a prospective longitudinal study of normoalbuminuric people with diabetes mellitus showed that the CKD273-model predicted progression to macroalbuminuria (the hallmark of diabetic nephropathy) 3-5 years earlier than did the current clinical screening method, microalbuminuria (Zurbig *et al.*, 2012).

8.4 Clinical implications

8.4.1 Epidemiology of kidney disease

Knowledge of breed differences regarding the occurrence of kidney disease may be of help in the diagnostic reasoning process in the clinic. In paper I, KD was estimated to have affected about 1.6 % of insured dogs over a 12 year period, based on data from the largest animal insurance company in Sweden. This estimate is in line with a previous suggestion regarding overall (point) prevalence (of CKD only; 0.5-1.5%), but both are probably conservative estimates because of the insensitivity of routine diagnostic methods for detection of both AKI and CKD (Brown, 2007). For a number of breeds, the entire 95% CI of both IR and MR were above the overall (total) IR and MR of KD: Bernese

mountain dog, boxer, Shetland sheepdog, miniature schnauzer, flat-coated retriever, soft-coated retriever, Rottweiler, cairn terrier and cavalier King Charles spaniel.

The Swedish elkhound, German wiredhaired pointer, mixed breed and Finnish spitz were the breeds for which the 95% CI of both IR and MR of KD was below the overall (total) IR and MR, respectively.

This breed analysis is not to be considered an exhaustive investigation, because for many (numerically smaller) breeds, there were not enough dogs insured at Agria over the years 1995-2007. Including breeds with fewer registered individuals could have produced very inaccurate results regarding single breed IR and MR.

8.4.2 Evaluation of cardiovascular function in CKD patients

An increase in circulating volume (as assessed by a calculated factor related to plasma volume) was shown to be associated with a decreasing mGFR in the dogs of paper II. As a probable consequence, NT-pro-BNP concentrations increased. In 43 of the 50 dogs in paper II (including all healthy dogs), NT-proBNP concentration was <900 pmol/L, which should be interpreted as "low risk of heart failure" according to information provided by the laboratory. In four dogs, NT-proBNP concentrations were within the 900 and 1800 pmol/L range, referred to as a grey zone by the laboratory. In three dogs, concentrations of NT-proBNP were increased (2167, 3173 and 7274 pmol/L) above the cut-off value (1800 pmol/L) that signifies a high suspicion of heart failure. The dog with the highest NT-proBNP concentration was diagnosed with protein-losing nephropathy, IRIS stage 3 CKD. All dogs were free of significant heart disease.

Assessment of NT-proBNP is probably not of value for routine clinical investigation of dogs with suspected CKD. Rather, the interpretation of this study is that NT-proBNP can be used for evaluation of cardiovascular compromise in dogs with a diagnosis of CKD. Interpretation of a single NT-proBNP-result in a dog needs consideration of different possible reasons for increased circulating volume. In addition to this, concentrations in healthy dogs vary considerably between breeds and between individuals (Ruaux *et al.*, 2015; Sjostrand *et al.*, 2014).

There was no association between cTnI concentration and mGFR, and no evidence for passive accumulation of cTnI concentration with decreasing mGFR. The conclusion was that cTnI reflects myocardial cell damage similarly in dogs with or without CKD. The interpretation of results of cTnI-analysis, when used for assessment of possible myocardial cell damage, need therefore probably not be different in dogs with stable CKD than in other dogs in a clinical

situation. The situation may be different for dogs with acute deterioration of renal function, regarding both NT-proBNP and cTnI.

8.4.3 Glomerular filtration rate assessment

The similar overall diagnostic value of SDMA, and the inferior diagnostic value of cystatin C, compared to creatinine indicate that creatinine can still be used as the first-line indirect marker of GFR. Whether one marker or the other is able to detect decreased GFR at an earlier point in time during disease development, depends on where the cut-off value is placed. A low cut-off value will result in decreased specificity and more false positive results. Conversely, a high cut-off value will result in lower numbers of "falsely azotaemic" dogs, but the sensitivity of the test will be lower and some dogs with decreased GFR will be missed. Both SDMA and cystatin C have potential as complementary tests to creatinine however, especially for selected dogs.

Creatinine concentrations should be interpreted with size of muscle mass of the dog in mind. If dogs are very small or have subnormal lean body mass (in which case use of a population-based reference interval is likely to result in decreased sensitivity of creatinine, or many false negatives) or large (in which case use of a population-based reference interval is likely to result in decreased specificity, or many false positives), the addition of another GFR marker such as SDMA or cystatin C concentration, may provide important additional information. As always, concerning estimations or calculations of GFR, any diagnosed decrease may represent a pre-renal, renal or post-renal, acute or chronic abnormality (or any combination of these).

Even if a perfect biomarker for GFR was discovered, or if a perfect method for direct measurement of GFR was invented – the inherent variability and the resulting wide reference range of GFR itself has to be taken into account when aiming to diagnose a small decline in GFR following a single measurement in an individual dog. The decrease in GFR is also a comparably late event in the gradual nephron destruction cycle that characterises CKD, as noted in section 8.3.2.

8.4.4 Urinary capillary electrophoresis and mass spectrometry

At present, there is no clinically available test that detects ongoing renal pathology at one point in time. Such tests are needed, and searched for, because of their potential for early diagnosis of progressive, or subclinically "active" disease. Urinary peptidomics analysis by CE-MS is a technique with potential as an adjunct diagnostic method for future non-invasive early diagnosis of CKD

in the dog. However, further validation of the diagnostic models 133P and 35P, described in paper IV, are necessary before possible future implementation. Although not yet commercially available, CE-MS is well suited for future clinical use because of high throughput and low cost of analysis (Schiffer *et al.*, 2012).

8.4.5 Future perspectives

During the work with this thesis (especially in the collaboration with human CKD researchers), difficulties with regard to the currently used definition of canine CKD became evident. An advantage with this definition is that it encompasses the whole spectrum of disease (unlike the terms "renal insufficiency" and "renal failure" that are imprecise, interchangeably used, and only refer to late stages of disease). A negative aspect of the current definition, however, is that individuals with non-progressive disease are included. In many dogs with a CKD diagnosis, this "disease" is not going to develop into a clinical problem throughout their lifetime.

Some dogs that are diagnosed with stage 1 CKD may have a structural, non-progressive, abnormality. Some have persistent renal proteinuria, which, even if present >3 months, may resolve. Thus, in addition to the dogs with non-progressive, non-clinical disease, the current definition also may include dogs that are, at least clinically, "cured". This may generate unnecessary confusion among owners of affected dogs and attending veterinarians. The labelling of a dog that has a non-progressive renal abnormality with a diagnosis of CKD may introduce unnecessary worry for owners and lead to a change in how the dogs are held, for example how the dog is exercised. Also, unnecessary and costly followup procedures have economic consequences for owners and insurance companies. Overdiagnosis is a term used in human medicine to describe a scenario in which the assignment of a diagnosis of disease leads to more harm than benefit for patients (Brodersen *et al.*, 2018; Heneghan *et al.*, 2017).

A definition of canine CKD that includes only dogs with irreversible, progressive disease is needed. Progression, if not obvious at the time of the first examination, currently has to be confirmed by serial testing. During the time interval (even if > three months) from identification of a kidney-related problem to the time-point of confirmation of progression, the dog may be considered to be at risk for [progressive] CKD, rather than to be assigned a CKD diagnosis. In accordance, a new category may be introduced for dogs "at risk" for CKD. When progression is confirmed, the dog could be given a definitive diagnosis of CKD, and staged accordingly into IRIS stages 1 through 4. This suggested definition should be thoroughly evaluated for its clinical use before implementation.

Should a diagnostic test that reliably detects progression become available in the future (for example the urinary peptidomics approach with the 133P-model investigated in paper IV), it will probably facilitate diagnosis according to this definition.

Table 2. *Suggested clinical definition of canine CKD, including IRIS staging*

	CKD			
At risk	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**
Structural or functional damage ≥ 3 months	Structural or functional damage ≥ 3 months Progression confirmed Creatinine: <125 µmol/L < 1.4 mg/L	Structural or functional damage ≥ 3 months Progression confirmed Creatinine: 125–180 1.4–2.0	Structural or functional damage ≥ 3 months Progression confirmed Creatinine: 180-440 2.1-5.0	Structural or functional damage ≥ 3 months Progression confirmed Creatinine: >440 µmol/L > 5.0 mg/L

9 Conclusions

- The incidence rate of KD in a large population of insured Swedish dogs was 15.8 cases/10.000 DYAR. The kidney-related mortality rate was 9.7 deaths/10.000 DYAR. The Bernese mountain dog was the breed with the highest incidence- and mortality rate of KD.

- In dogs with and without CKD, the variables independently associated with NT-proBNP was PCV and PVF/kg, a factor related to extracellular fluid volume. Age, BW and SBP were the factors independently associated with cTnI concentration. Neither NT-proBNP nor cTnI concentrations were independently associated with mGFR. Results were not suggestive of passive accumulation of NT-proBNP or cTnI in clinically stable dogs with CKD.

- Overall diagnostic performance of SDMA concentration as a marker of decreased GFR in dogs was similar to that of creatinine but better than that of cystatin C. Use of SDMA and cystatin C in addition to creatinine conveyed additional value for prediction of mGFR status in clinically stable dogs.

- A model, 133P, was developed by CE-MS-based urinary peptidome analysis, and was shown to discriminate dogs with CKD from healthy dogs in a separate validation cohort (AUC = 0.88). Thirty-five out of the 133 peptides contained in the model were sequenced, and most were identified as fragments of collagen I.

10 Implications for future research

10.1 Epidemiology of canine chronic kidney disease

Further studies of incidence and mortality rates of CKD should be performed in other populations of dogs. Differences between breeds should be investigated in order to possibly confirm the high IRs in some breeds identified in paper I. Qualitative studies involving questionnaires directed at dog owners could possibly also contribute to knowledge regarding impact of KD in different dog populations.

10.2 Cardiovascular biomarkers in kidney disease

Interactions between the kidneys and the cardiovascular system in CKD need further investigation. The conclusion in paper II regarding GFR-independence of both NT-proBNP and cTnI should be confirmed in other populations of dogs with CKD, and in dogs with acute-on-chronic disease and AKI. A possible value of NT-proBNP concentration for estimation of circulating blood volume in some dogs with CKD (for example protein-losing nephropathy and nephrotic syndrome) is also called for.

10.3 Indirect assessment of glomerular filtration rate

Diagnostic performance of SDMA concentration as a marker of decreased GFR should be further studied in other populations of dogs. The interval LRs proposed in paper III needs further validation in such studies. The value of performing additional tests (SDMA and cystatin C concentrations) in dogs for which serum creatinine concentration is suspected to be a suboptimal test of GFR, should be prospectively investigated.

A new SDMA test for use in the clinic is released in 2018. Evaluation of the diagnostic performance of this benchtop analyser test as a marker of GFR in the dog is needed, especially in light of the high analytic variability shown for a variety of bench-top analyses of creatinine concentrations in one study (Braun *et al.*, 2008). Additionally, RCV and individuality of SDMA (as well as creatinine and cystatin C) needs to be established, preferably by evaluating dogs of many different breeds and sizes.

Cystatin C holds potential as a marker of GFR in the dog. The value of this test as an additional marker of GFR, using the proposed decision tree and the interval LRs proposed in paper III, needs further validation. A reference interval should be constructed for the cystatin C-analysis method used in paper III. The age-dependence of cystatin C identified in paper III should be further investigated in other populations.

10.4 Urinary peptidomics

Further validation of the diagnostic value of the 133P-model in other canine cohorts is necessary. Although a high repeatability has been shown for CE-MS analyses of human urine, repeatability for canine urine samples should be assessed. Variations, pre-analytical, analytical and biological variation highly affect clinical utility of an assay. Such investigations should include longitudinal studies of dogs with and without CKD.

Specificity of the model needs to be addressed. The ability to distinguish dogs with CKD from dogs with other diagnoses is not known but should be investigated. This is especially important for diagnoses that are regarded differential diagnoses to CKD in a clinical situation, such as hyperadrenocorticism, diabetes insipidus or primary polydipsia. Early diagnosis of renal compromise by proteomic methods may be also complicated by changes that occur in renal ageing. Therefore, peptidomic models should be constructed to distinguish a pathological process from the aging process that is bound to occur.

Investigation of the performance of the 133P-model in prediction of progression of canine CKD is to be performed. Should the 133P-model be able to detect the progressive fibrosing process itself, assessment of progression would be markedly facilitated in the clinic.

Application of this technique for differential diagnosis of various aetiologies of CKD, potentially lessening the need for renal biopsy, is another future goal. Performing renal biopsy requires an invasive, expensive approach, carries a risk, and is therefore often not performed. In addition, the resulting small piece of tissue is not necessarily representative of the global tissue mass and the biopsy

procedure is seldom repeated for the reasons listed above. Value of the CE-MS approach in differentiating aetiologies of CKD has been shown recently in people (Siwy *et al.*, 2016). Urinary peptidomics analysis may also be more representative of global tissue pathology compared to a biopsy specimen. An association between CE-MS peptide pattern and tissue histopathology has been demonstrated in one study (Magalhaes *et al.*, 2017). Use of CE-MS in this respect has been proposed to represent a form of "liquid biopsy". This should be studied in dogs as well, by comparing peptide patterns in urine from dogs with different specific aetiologies of CKD.

Further identification and sequencing of peptides associated with development of canine CKD may provide insights regarding specific pathophysiologic events involved in progression. Identification of relevant proteases responsible for cleavage of fragments involved in the process of fibrosis could possibly lead to future studies of the efficacy of proteinase-inhibitor treatment, as shown in a proof-of-concept study in people with diabetic nephropathy (Krochmal *et al.*, 2017).

Finally, it has been shown in people that the peptidomic profile changes in response to treatment (Andersen *et al.*, 2010a). This could be investigated in dogs as well, because if it applies also for dogs, and if efficacy of the investigated treatment is proven, peptide profiling may represent a future tool for monitoring treatment with the target of "normalising" the peptide profile.

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11 Popular science summary

Chronic kidney disease (CKD), which is an important contributor to morbidity and mortality in dogs, is defined as the presence of structural or functional abnormalities in one or both kidneys for > three months. There are multiple aetiologies of CKD, but in many dogs the initiating cause is not evident (and may no longer be present) at the time of diagnosis. Clinical signs associated with decreased kidney function include loss of appetite and body weight, vomiting, and increased urine production and water intake. Current veterinary diagnostic methods are insensitive, and new markers for early disease detection are sought. The general aim of this thesis was to increase knowledge regarding pathophysiologic mechanisms and early diagnosis of canine CKD, by identifying dogs with increased risk of disease and by exploring the use of various biomarkers in blood and urine.

Dogs are comparably commonly diagnosed with kidney-related disease (KD). A conservative estimate of the prevalence (percentage of dogs that are affected) of CKD has been suggested to be 0.5-1.5%. Incidence (how many cases that are diagnosed) or mortality (how many deaths that occur) rates of KD are not known for either acute or chronic presentations. Such knowledge is, however, of practical use in clinic and research. Accordingly, the aim of paper I was to use data from a large animal insurance company database (Agria Animal Insurance) to estimate incidence and mortality related to kidney disease in a Swedish population of >600,000 insured dogs. The total incidence rate of kidney disease in this population was 15.8 cases per 10,000 dog years at risk (DYAR, a denominator representing one dog insured for one year). The breeds most commonly diagnosed with KD in this study were the Bernese mountain dog, miniature schnauzer and boxer. The Swedish elkhound, Siberian husky and Finnish spitz were the breeds least often diagnosed with KD.

The cardiovascular system and the kidneys interact extensively in both health and disease. Increased concentrations in plasma or serum of two cardiovascular biomarkers, B-type natriuretic peptide (NT-proBNP) and cardiac troponin I

(cTnI), have been reported in dogs with decreased renal function. A passive accumulation of these markers in dogs with disease of the kidneys has been suggested. The aim of paper II was therefore to investigate if NT-proBNP and cTnI accumulate in dogs with CKD. Results presented in paper II do not support passive accumulation of these markers. Instead, the conclusion was that they identify pathophysiological events (increased blood volume and damage to cardiac cells, respectively) in some dogs with CKD.

Considerable effort is directed at identification of new diagnostic circulating markers of decreased renal function (glomerular filtration rate, GFR) for widespread clinical use in veterinary medicine. Symmetric dimethyl arginine (SDMA) and cystatin C are two proposed biomarkers of decreased GFR in the dog. In paper III, the aim was to investigate overall diagnostic value of serum SDMA and cystatin C concentrations for detection of decreased GFR compared to the currently most commonly used marker, serum creatinine concentration. The overall diagnostic value of SDMA was equivalent to that of creatinine, but cystatin C performed less well as a marker of decreased renal function in this study. Combining markers appeared to be of diagnostic value for diagnosis of a decreased GFR.

In human medicine, urinary peptidomics may be utilised to diagnose CKD at an early stage. A specific urinary peptide pattern (CKD273-model) that detects CKD has been developed and shown to be of value both for diagnosis of CKD and for prediction of deterioration of renal function over time in people. Changes in the canine urinary peptide pattern may represent a completely new opportunity for early diagnosis of CKD as well. The usefulness of urinary peptidomics as a diagnostic method has not previously been investigated in dogs. In paper IV, it was shown that a CE-MS-based urinary peptidome model, 133P, was able to discriminate healthy dogs from dogs with CKD in a separate validation-cohort. This model, although in need of further investigation and validation, represents an exciting new diagnostic modality, in that it may potentially detect chronic progressive CKD in a single urine sample.

Results of the studies included in this thesis contribute with knowledge relevant to the diagnostic thought process in the evaluation of dogs with suspected kidney disease, and with new information regarding interactions between kidneys and the cardiovascular system. Also, a new diagnostic modality for CKD diagnosis is presented that may, if found useful in further studies, make a contribution to canine renal medicine in the future.

12 Populärvetenskaplig sammanfattning

Kronisk njursjukdom (CKD) är en allvarlig sjukdom hos våra hundar. Många olika underliggande orsaker till CKD finns beskrivna. Då sjukdomen många gånger upptäcks jämförelsevis sent i sjukdomsförloppet är den exakta orsaken ofta inte möjlig att fastställa, eller åtgärda, vid tidpunkten för diagnos. De övergripande målen med denna avhandling var att öka kunskapen kring patofysiologiska mekanismer vid, och tidig diagnostik av, CKD hos hund.

Njursjukdom förekommer förhållandevis ofta hos våra hundar, men uppgifter saknas avseende hur ofta njursjukdom diagnosticeras och hur ofta det leder till att hundar dör. I studie I sammanställdes data från > 600 000 hundar registrerade hos Agria Djurförsäkring under en tolvårsperiod (1995-2007). Förekomst av, samt dödlighet till följd av, njursjukdom undersöktes. Förekomsten beräknades till ca 16 nya fall per 10 000 hundår (ett mått som innebär en hund försäkrad under ett år). Dödligheten till följd av njursjukdom uppgick till ca 10 dödsfall per 10 000 hundår. Information togs även fram för de i databasen vanligast förekommande raserna. Högst förekomst av njur-relaterad sjukdom sågs hos berner sennen, dvärgschnauzer och boxer. Lägst förekomst sågs hos jämthund, siberian husky och finsk spets. Många raser kunde dock inte inkluderas i denna jämförelse, på grund av ett för litet antal försäkrade hundar inom rasen.

B-type natriuretic peptide (NT-proBNP) och hjärtspecifikt troponin I (cTnI) utgör två sjukdomsmarkörer som kan mätas i blodet och används för att upptäcka ökad cirkulerande blodvolym respektive hjärtmuskelcellskada hos hund. Tidigare studier har visat att höga koncentrationer av dessa markörer kan ses i blodet hos hundar med njursjukdom. Orsaken till detta har inte varit känd. En passiv ackumulering av dessa ämnen i blodet, vilket skulle medföra att deras diagnostiska värde för detektion av hjärt- och kärlpåverkan minskar har tidigare inte kunnat uteslutas, och detta undersöktes i studie II. Resultaten indikerade frånvaro av passiv ackumulering i blodet, och slutsatsen var att dessa markörer indikerar hjärtmuskelcellskada och ökad cirkulerande blodvolym hos hundar såväl med som utan CKD.

Inom veterinärmedicinen pågår ett ständigt sökande efter nya diagnostiska metoder för detektion av nedsatt njurfunktion i ett tidigt skede. Symmetrisk dimethylarginin (SDMA) och cystatin C är två potentiella markörer som kan användas för detektion av nedsatt njurfunktion. I studie III jämfördes det diagnostiska värdet av dessa markörer, jämfört med värdet av den markör som används sedan länge för diagnos av nedsatt njurfunktion; koncentrationen av kreatinin. Det visade sig att det övergripande diagnostiska värdet av SDMA för diagnos av nedsatt njurfunktion var likvärdigt med värdet av kreatinin. Det övergripande värdet av cystatin C för diagnos av nedsatt njurfunktion var dock lägre än värdet av de båda andra markörerna. För vissa hundar finns dock troligen ändå anledning att analysera koncentrationer av SDMA eller cystatin C förutom koncentrationen av kreatinin, för att erhålla en förbättrad utvärdering av njurfunktionen.

I studie IV utvärderades en helt ny metod (kapillär elektrofores och masspektometri, CE-MS), för diagnosticering av njursjukdom. Med hjälp av denna metod undersöks urinens hela innehåll av proteinfragment, så kallade peptider. Skillnader i peptidmönster mellan sjuka och friska individer undersöktes i studien och en statistisk modell, 133P, för diagnostik av CKD konstruerades. Validering av 133P-modellen i en separat grupp av hundar med och utan CKD visade att modellen kunde särskilja dessa grupper. Urinanalys med hjälp av CE-MS behöver valideras ytterligare, men utgör ett spännande nytt diagnostiskt alternativ för tidig diagnostik av CKD hos hund i framtiden.

Resultat från de studier som ingår i denna avhandling bidrar med kunskap som kan vara till nytta i den diagnostiska tankeprocessen samt med ökad förståelse för hjärt- och kärlpåverkan hos hundar med CKD. Dessutom presenteras en ny diagnostisk metod som, om den håller måttet vid utvärdering i framtida studier, potentiellt kan komma att göra stor skillnad inom veterinär njurmedicin i framtiden.

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