INVESTIGATION OF CYSTATIN C FOR THE DETECTION OF FELINE CHRONIC KIDNEY DISEASE

Liesbeth Ghys

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Department of Medicine and Clinical Biology of Small Animals

Faculty of Veterinary Medicine

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Promotor: Prof. Dr. Sylvie Daminet

Co-promotor: Prof. Dr. Dominique Paepe

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ABBREVIATIONS

^{51}Cr-EDTA	Chromium ethylenediamine tetra-acetic acid
^{99m}Tc-DTPA	Technetium diethylenetriamine penta-acetic acid
ACVIM	American College of Veterinary Internal Medicine
ACE	Angiotensin-converting enzyme
ADH	Antidiuretic hormone
AKI	Acute kidney injury
ARB	Angiotensin receptor blocker
ASVCP	American Society of Veterinary Clinical Pathology
BC	Serum biochemistry
BCS	Body condition score
BUN	Blood urea nitrogen
CBC	Complete blood cell count
CKD	Chronic kidney disease
Cr	Creatinine
CRP	C-reactive protein
CysC	Cystatin C
CV	Coefficient of variation
Da	Dalton
DM	Diabetes mellitus
DLH	Domestic longhair cats
DSH	Domestic shorthair cats
ELISA	Enzyme-linked immunosorbent assay
FIV	Feline immunodeficiency virus
GLM	General linear modelling
GM-CSF	Granulocyte-macrophage colony stimulating factor
GFR	Glomerular filtration rate
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IoI	Index of individuality
IL-8	Interleukine-8
IRIS	International renal interest society
kDa	Kilo Dalton
LMW	Low molecular weight
LOD	Limit of detection
MM	Molecular mass
NAG	N-acetyl-β-D-glucosaminidase
NSAID	Non-steroidal anti-inflammatory drug
PCR	Polymerase chain reaction
pCysC	Plasma cystatin C
PEC-ICT	Plasma exogenous creatinine-iohexol clearance test
PECCT	Plasma exogenous creatinine clearance test

PenICT	Plasma endo-iohexol clearance test
PENIA	Particle enhanced nephelometric immunoassay
PETIA	Particle enhanced turbidimetric immunoassay
PexICT	Plasma exo-iohexol clearance test
PICT	Plasma iohexol clearance test
RAAS	Renin-angiotensin-aldosterone system
RBP	Retinol binding protein
RI	Reference interval
ROC	Receiving-operating-characteristic
SBP	Systolic blood pressure
sCysC	Serum cystatin C
sCr	Serum creatinine
SD	Standard deviation
SDMA	Symmetric dimethyl arginine
TGF-β1	Transforming growth factor-β1
TT4	Total thyroxine
uCr	Urinary creatinine
uCysC	Urinary cystatin C
uCysC/uCr	Urinary cystatin C: creatinine ratio
UPC	Urinary protein:creatinine ratio
USG	Urine specific gravity

CHAPTER I

GENERAL INTRODUCTION

This first chapter is divided in three parts.

In the first part, anatomy, physiology of the kidneys are briefly presented, followed by pathophysiology, symptoms and diagnosis of chronic kidney disease (CKD).

The second part summarizes current knowledge on cystatin C in human and veterinary medicine, namely its history, assays, relationship with glomerular filtration rate (GFR), biological and clinical variations both in human and veterinary medicine.

In the third part, the need for and different aspects of validation to understand the scientific aims of this thesis, described in Chapter II.

1. Feline chronic kidney disease

1.1. Anatomy of the feline kidney

The kidneys are well-protected bean-shaped paired organs located in the retroperitoneal space.[1] The normal size of feline kidneys is between 3.7 and 4.0 cm.[2] Macroscopically on dorsal section, every kidney consists of an outermost region, the cortex, an inner region, the medulla and a central area, the renal pelvis, with entrance, exit and origin of renal artery, renal vein and ureter respectively[1] (Figure 1, 2). The functional unit of the kidney is the nephron, which consists of the renal corpuscle, the renal tubules and the collecting duct (Figure 3). The renal corpuscle consists of the glomerulus and Bowman's capsule and is located in the cortex.[3] A feline kidney contains 175 000 nephrons.[4] Every part of the nephron has its specific function, which will be explained briefly in the following section.

Figure 1. Venous plastinate preparation of a feline kidney. (scale in cm) (Picture Bart De Pauw, museum of morphology, faculty of veterinary medicine, Ghent University)

Figure 2. Longitudinal cross-section of the kidney. (Picture Bart De Pauw, museum of morphology, faculty of veterinary medicine, Ghent University)

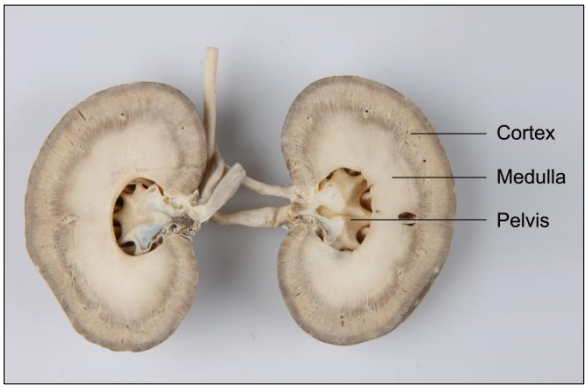
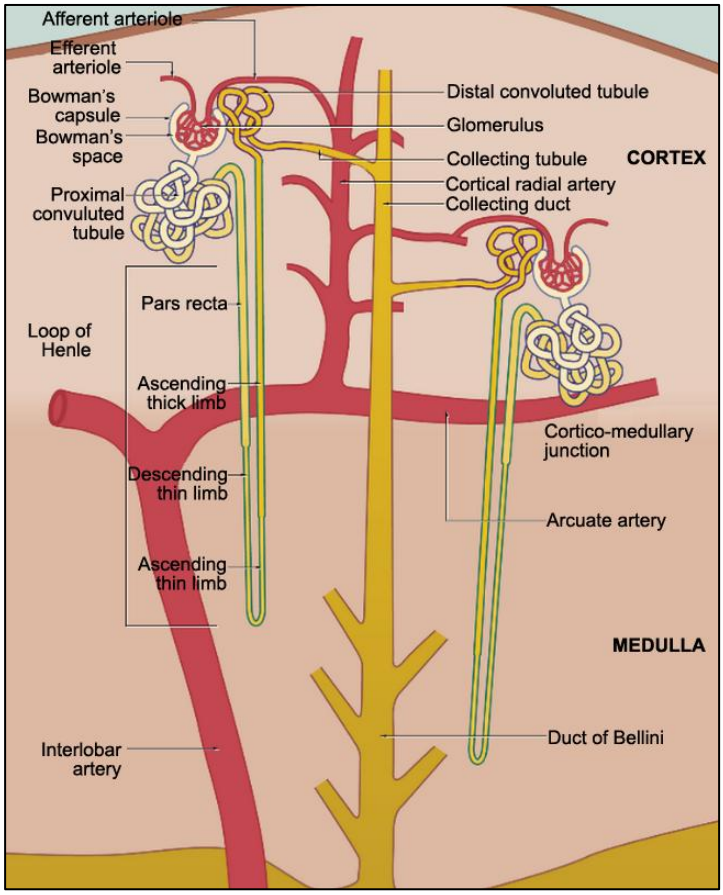


Figure 3. The nephron. Adapted from Young B et al., 2006.[5]



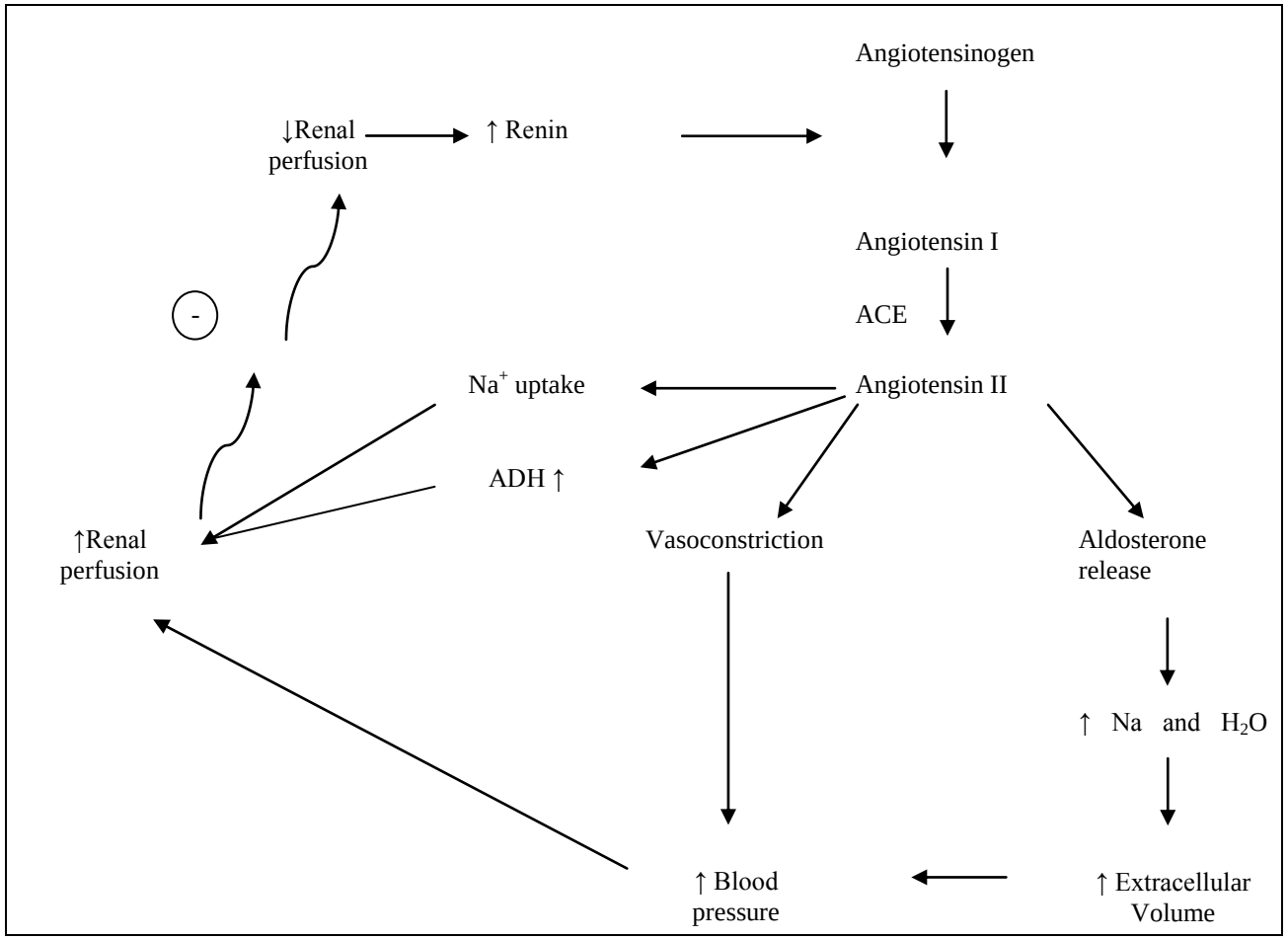
1.2 Physiology

The kidney is responsible for maintaining homeostasis. In mammals, both kidneys receive approximately 25% of the cardiac output. First, blood is filtered in the *glomerulus*, which consists of a network of capillaries enclosed within Bowman's capsule.[6] Glomerular filtration rate (GFR) is determined by the mean net filtration pressure, permeability of the filtration barrier and area available for filtration.[6] The main force favouring filtration is the glomerular hydrostatic pressure of cardiac origin opposed by plasma (oncotic) pressure of plasma proteins and urine hydrostatic pressure within Bowman's capsule.[6,7] The glomerular filtration barrier consists of the capillary endothelium, the glomerular basement membrane and the visceral epithelium with podocytes.[6] The permeability is determined by the size and the charge of the molecules within the bloodstream.[6] In normal kidneys, high and intermediate molecular weight proteins and the anionic form of the molecules are retained

within the blood stream, whereas water and solutes are freely filtered The glomerular filtrate is collected in Bowman's space and flows to the tubular lumen.[6]

The main function of the *tubules* is secretion and reabsorption of filtered substances. The tubules are divided into several segments, with every segment having a specific function. In the *proximal tubule*, at least 60% of most filtered substances is reabsorbed by several mechanisms including passive diffusion, solvent drag, primary active transport, and carrier-mediated secondary active transport.[6] Active tubular secretion of creatinine (Cr) occurs in the proximal tubule, in humans,[8] but is minor in dogs[9,10] and negligible in cats.[11] The *thin limb of the loop of Henle 's* major function is essential for urine concentration by water reabsorption.[6,7] The *ascending loop of Henle* and *the distal tubules* reabsorb salts without water,[7] and can secrete or reabsorb potassium.[6] In the *collecting duct* and the final segment of the distal tubules, solute excretion and final regulation of urine volume occur.[6,7] The reabsorption in the proximal tubule occurs independently of the physiologic state. In the distal tubule and collecting duct on the other hand, the processes are controlled by several hormones, including aldosterone, angiotensin II, antidiuretic hormone, endothelin, atrial natriuretic peptide, parathyroid hormone, calcitriol and calcitonin. Aldosterone and angiotensin II are both part of the renin-angiotensin-aldosterone system (RAAS), increasing blood systemic and glomerular pressure, and therefore increasing GFR. The mechanism is explained in Figure 4. Renin is released after a decrease in renal perfusion and catalyses the transformation of angiotensinogen to angiotensin I, which is converted to the more active angiotensin II by angiotensin-converting enzyme (ACE). Angiotensin II activates sodium uptake in both the proximal tubule and collecting duct and stimulates the release of aldosterone and vasopressin. Aldosteron enhances Na^+- and water reabsorption to correct volume depletion.[5] Antidiuretic hormone (ADH, vasopressin), enhances salt reabsorption from the thick ascending limb and the collecting duct, and does not enhance dilution of the tubule fluid, but water reabsorption in the collecting ducts.[5] Endothelin and atrial natriuretic hormone regulate sodium transport in the distal part of the nephron. Parathyroid hormone, calcitriol and calcitonin regulate calcium reabsorption in the distal nephron and connecting segment.[6] The kidney itself also produces two hormones: erythropoietin and calcitriol. Erythropoietin is synthesized in the peritubular cells, and regulates erythrocyte production in response to hypoxia. Calcitriol induces active intestinal calcium absorption and acts in synergy with parathyroid hormone for calcium release from bone.[7]

Figure 4. Schematic overview of the renin-angiotensin-aldosterone system. Adapted from Verlander J, 2007.[6]



1.3 Feline chronic kidney disease

1.3.1 Definition, prevalence and cause

Chronic kidney disease (CKD) is characterised by a permanent reduction in functioning nephrons. Compensatory mechanisms try to sustain kidney function, but lead to a progressive loss of remaining functioning nephrons.[12] Therefore, CKD is an irreversible and slowly progressive disease. It is defined as kidney damage that has existed for at least 3 months, with or without reduction in GFR for more than 3 months.[12] The estimated prevalence in cats is between 1.6 and 20%,[13] and increases with age, with over 30% in cats older than 15 years.[14] Breeds more often represented are Siamese, Abyssinian, Persian, Maine Coon and Burmese cats.[13-16]

Chronic kidney disease can be caused by several diseases and disorders that affect glomeruli, tubuli, blood supply or the interstitium. However, damage to each of these components affects the entire nephron.[17] In cats, tubulo-interstitial nephritis is most commonly observed.[12] Causes of CKD can be congenital or acquired, but the initiating cause can often not be determined at time of diagnosis.[12]

1.3.2 Consequences and symptoms

Since the kidneys are involved in homeostasis of the entire body, CKD affects many organs, and therefore general condition.[18] Symptoms manifest due to retention of products that should normally be excreted (e.g. phosphorus, urea, Cr) or excretion of compounds that should normally be retained (e.g. water and protein).[18] *Polyuria and polydipsia* are caused by a decrease in adequate urine concentrating ability due to increased solute load per surviving nephron and loss of responsiveness to ADH.[12] *Azotemia* is defined as increased nonprotein nitrogenous compounds in the blood, detected as increased serum urea and serum Cr (sCr).[14] *Uremia* refers to the clinical signs related with renal failure e.g. anorexia, nausea, halithosis (uremic breath), diarrhea, melena and convulsions.[12,19] Uremia is caused by disturbed excretion of electrolytes and water, reduced excretion of organic solutes and impaired hormone synthesis.[12] *Systemic hypertension* is common in feline patients with CKD and may cause CKD or may adversely affect renal function.[20] CKD causes hypertension by retention of sodium, activation of RAAS, vascular dysfunction and endothelin production.[21] On the other hand, systemic hypertension might cause glomerular hypertension and proteinuria,

retinopathy or choroidopathy, encephalopathy and left ventricle hyperthrophy.[22] *Renal secondary hyperparathyroidism* is an increased production of parathyroid hormone with parathyroid cell proliferation, due to hypocalcemia, hyperphosphatemia and reduced renal calcitriol (vitamin D_3) production.[7,20] Clinical signs are rather uncommon in cats,[12] but it is essential to monitor phosphorus since parathyroid hormone is an uremic toxin and a major contributor to CKD progression.[7] *Metabolic acidosis* results from loss of ability to excrete hydrogen ions and decreased filtration of acids.[12] It has been shown that acidosis is a consequence rather than a cause of CKD progression. Clinical effects include anorexia, nausea, vomiting, lethargy, weakness, muscle wasting, weight loss and loss of appetite (Figure 5).[23] *Anemia* is mainly caused by erythropoietin deficiency, but iron deficiency due to malnutrition, myelofibrosis, erytropoietic inhibitor substances and blood loss might contribute also.[12] Clinical signs include pale mucous membranes, weakness and anorexia.[12] *Hypokalemia* is caused by decreased intake, increased loss or translocation of fluid from extracellular to intracellular.[24] The most common symptom in cats is polymyopathy, but mild cardiac arrhythmias can also occur.[12] On histopathology, the kidneys are small, irregular and pitted. The asymmetry between the kidneys may reflect differences in severity of the disease and the effect of compensatory hypertrophy (Figure 6).[12]

Figure 5. Cat with CKD (IRIS stage 4). Note the thin body condition score (1/9), muscle atrophy of the hindlimbs and spine (→), and bad hair condition.

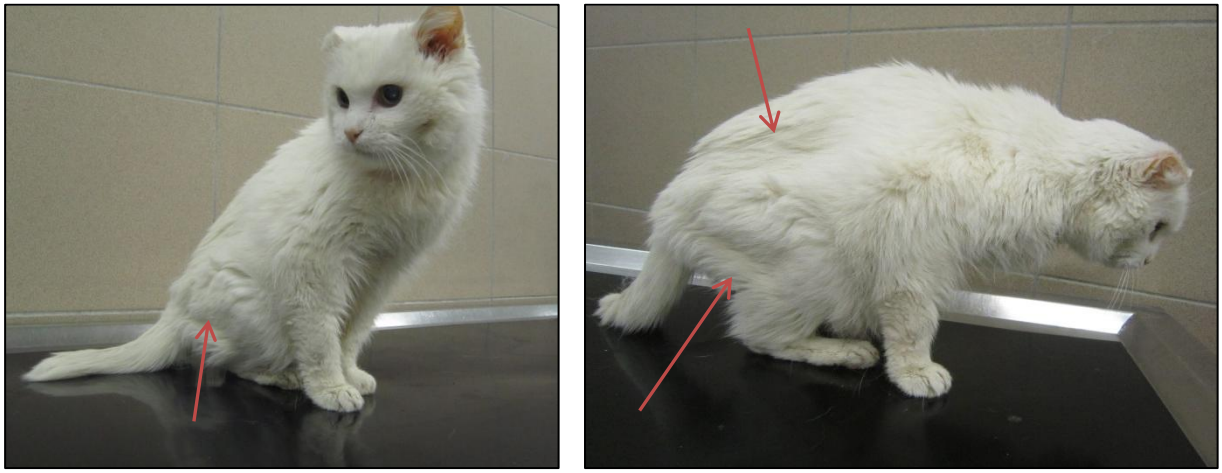
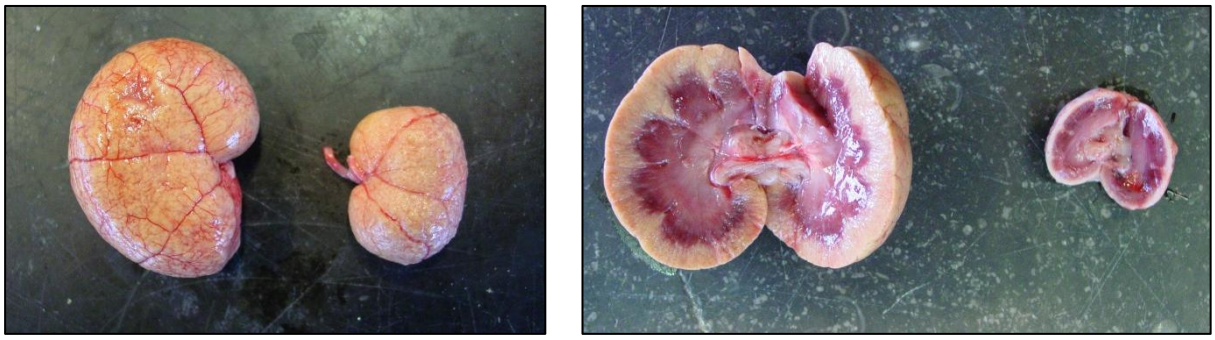


Figure 6. Kidneys from a cat with end stage renal failure. (Pictures Leslie Bosseler, department of pathology, bacteriology and poultry diseases, faculty of veterinary medicine, Ghent University)



1.3.3 Progression and survival of feline chronic kidney disease

Due to compensatory hypertrophy of the remaining nephrons, clinical signs and laboratory abnormalities compatible with CKD only manifest in an advanced stage of the disease, limiting therapeutic options to palliative or supportive care.[17,25] The standard care for feline CKD is aimed at improving clinical signs and slowing down disease progression.[26] Cats with CKD that were treated with a renal diet, characterized by a reduced protein, phosphorus and sodium content had less uremic episodes and showed a significant reduction in renal-related death.[27] In contrast to treatment with a renal diet, no survival benefit from treatment of proteinuria with benazepril, an ACE-inhibitor was demonstrated.[28] Although recent International Renal Interest Society (IRIS) guidelines recommend treatment of CKD cats with urinary protein:creatinine ratio (UPC) $\geq$ 0.4 in all IRIS stages – also in IRIS stage 1 –, the point at which a reduction in proteinuria improves survival is currently unknown.[29] The higher Cr, urea, phosphate, UPC and lower haematocrit at time of diagnosis, the shorter the survival time is in cats with CKD.[26,28,30,31] Consequently, it was demonstrated that cats diagnosed and treated in an earlier stage of the disease have an increased median survival time.[26] It is suspected that early treatment can delay progression of kidney dysfunction and prevent or postpone disease complications.[17,25] However, at this point, no renal variable can reliably predict the survival time in a cat with CKD.[20]

1.4 Diagnosis

1.4.1 Routine diagnosis of feline chronic kidney disease

1.4.1.1 Signalment, history, physical examination

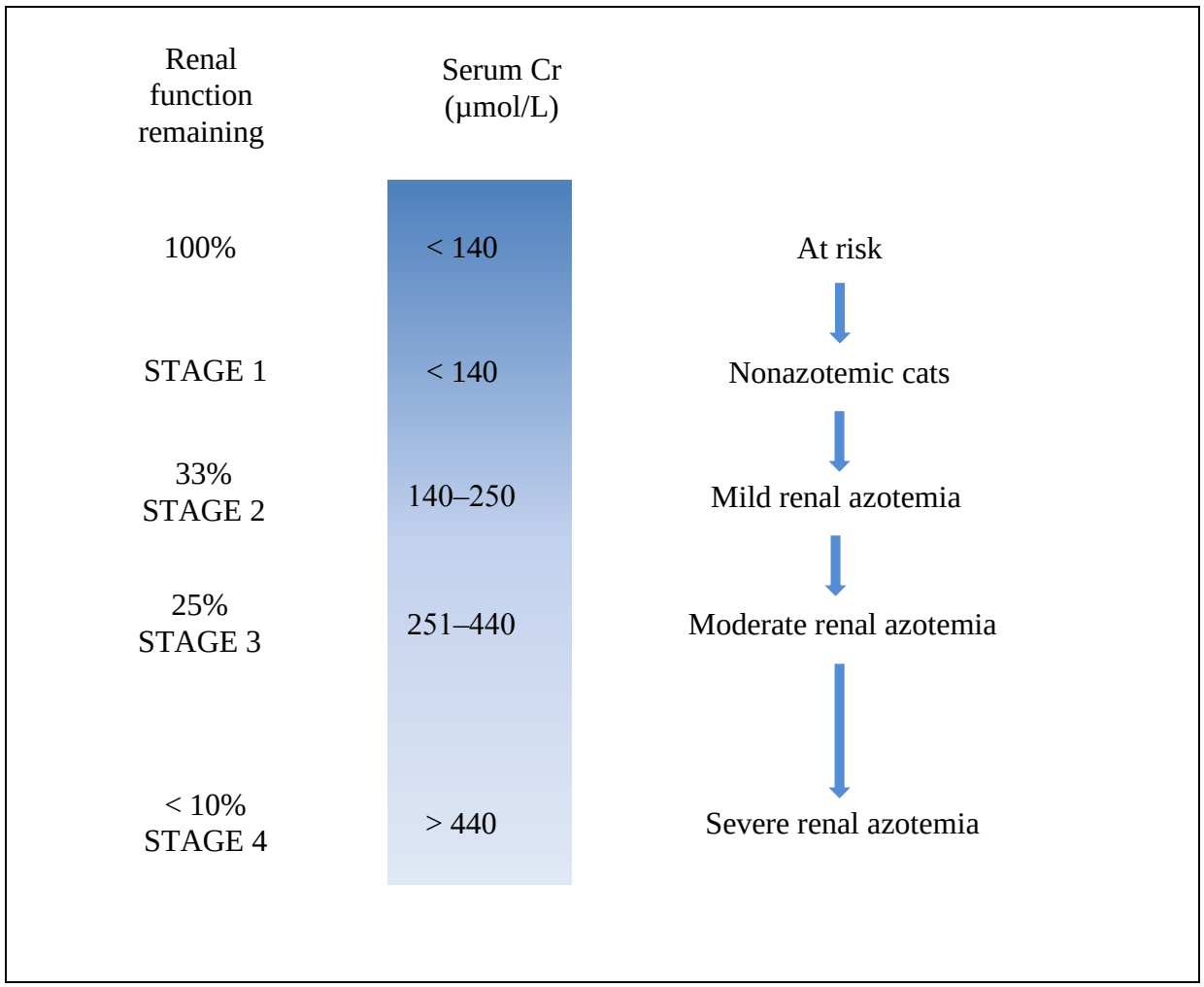
Nevertheless breed disposition has been described for underlying renal diseases, and most cats are geriatric, CKD can manifest in cats from all ages and breeds.[12,16] There is also a large variation in the stage of illness in which cats are presented to the veterinarian.[16] The most commonly observed historic findings are polyuria and polydipsia, lethargy, anorexia and weight loss. The abnormalities on physical examination include dehydration, mostly small and irregularly shaped kidneys, poor body condition score, and periodontal disease. The signalment, history and clinical findings can give an indication for CKD, but are nonspecific, making diagnosis of CKD difficult.[15,16] In addition, in early stages of CKD, no clinical signs or abnormalities on physical examination are present.[15,16] Therefore, blood and urine sampling are necessary to diagnose CKD.

1.4.1.2 Azotemia

Azotemia is routinely measured by serum urea and sCr.[25,32] Urea is the main form of nitrogen elimination from protein catabolism.[32] It is freely filtered by the glomerulus and reabsorbed by the tubules. Creatinine concentration in blood is determined by production of Cr by the muscles and renal excretion,[25] but also on the volume of distribution, demonstrated in dogs.[9] Creatinine is produced by degradation of creatine and creatine-phosphate, mainly present in skeletal muscles.[7] Therefore, production is proportional to muscle mass and might change due to muscle wasting.[25] Creatinine is almost completely excreted by relatively constant glomerular filtration without tubular reabsorption or secretion in cats.[11,32] Since Cr production is rather constant, GFR can be estimated based on sCr concentration. In contrast to sCr, urea production is not constant and is more heavily influenced by extra-renal factors than sCr, making urea not as useful as indicator of GFR.[7,32] A major limitation of both urea and sCr is the low sensitivity to detect kidney dysfunction. The relationship between GFR and urea and Cr is inverse curvilinear, which means that changes in Cr and urea are only small in early stages of CKD. It is assumed that approximately 75% of the nephrons must be non-functional until sCr or urea increase above the upper limit of the reference interval (RI) (Figure 7).[7,12] Measurement of urea is useful for monitoring the effect of dietary protein restriction.[33]

Urea is mostly measured with an enzymatic assay.[7,32] For sCr, routine analyses in cats and dogs are mostly based on the nonspecific Jaffe reaction.[34] Jaffe does not represent an analytical method, but refers to a chemical reaction i.e. the red colour formation when Cr reacts with picric acid in an alkaline medium.[35] Pseudo-creatinine chromogens i.e. proteins, ketones and glucose, overestimate true sCr.[36,37] Some manufacturers have adjusted the Cr calibration i.e. the modified Jaffe, to minimize the pseudo-Cr contribution, but this strategy assumes that the pseudo-Cr chromogen interference is a constant among samples, which is an oversimplification.[36] The enzymatic method deals more effectively with the interferences,[38] but a small overestimation is possible compared with the former golden standard method, high performance liquid chromatography (HPLC).[39] In cats, the enzymatic method favours the (modified) Jaffe method, which overestimates low sCr and underestimates high sCr.[39] However, the results of that study have to be interpreted cautiously, because, to the authors' knowledge, no comparison of feline sCr measured with the (modified) Jaffe or the enzymatic method, with the new proposed golden standard in humans, the gas-chromatography-isotope dilution mass spectrophotometry (IDMS) reference method, has been performed.[36] In addition, the recent introduction of the global Cr standard material SRM 967 has influenced sCr values in humans, but there are no guidelines yet how to adapt sCr calibration for the different assays according to the IDMS standard.[35] No information in cats is currently available. Despite its limitations, sCr is the most efficient indirect GFR marker in mammals.[7] Another limitation of sCr are the inappropriate RIs. In the reference groups, it is possible that animals with unrecognized CKD are included.[25] According to the guidelines of the American Society of Veterinary Clinical Pathology (ASVCP), a RI should be determined in a healthy population, taking into account clinical, biological and geographical factors. Biological variability, including the breed effect and interference should be considered to determine the need for separate RIs.[40] In addition, RIs differ between laboratories, making interpretation of results difficult.[41]

Figure 7. Percentage of remaining renal functional capacity. Adapted from http://www.iris-kidney.com[42] (Accessed 20/10/2015).



1.4.1.3 Urine specific gravity

Urine specific gravity (USG) is the weight of urine compared with an equal volume of distilled water. It is an accurate reflection of urine osmolality and of the solute concentration of the urine solution. It is a reference standard to evaluate urine concentration.[32] Urine specific gravity is estimated by measurement of the refractive index with a refractometer. Most refractometers are calibrated for human urine, and may overestimate feline urine concentration.[19,43] However, errors do not change clinical decisions and are more pronounced for concentrated urine samples, which make the human devices useable.[43,44] Both digital and optical hand-held refractometers correlate well with feline urine osmolality, but it is recommended to always use the same assay, since USG measurements with the digital refractometer are consistently higher.[44] Urine specific gravity is lower in old cats and after water intake.[45] Feline urine may be highly concentrated or diluted, depending on the hydration status of the cat, and the USG RI is between 1.001 and 1.065 or even 1.080.[19,46] Based on comparison of urine solute concentration with glomerular filtrate, iso-, hypo- and hypersthenuria can be defined. *Isosthenuria* (USG 1.007–1.015) is defined as urine with the same, *hyposthenuria* (USG < 1.007) as urine with lower and *hypersthenuria* (USG >1.015) as urine with higher concentration as unaltered glomerular filtrate respectively.[32] Some cats with CKD can retain adequate urine concentrating ability in the early phase of the disease, but most cats with CKD have USG < 1.035.[14-16,34] Urine specific gravity is the major laboratory parameter for differentiating azotemia. In case of CKD, persistent renal azotemia is present, i.e. renal azotemia present in a fasted and well-hydrated pet over several weeks.[12,42] The three types of azotemia with determinants are presented in Table 1.

Table 1. Three types of azotemia with the major criteria to differentiate them. Adapted from Stockham SL and Scott MA, 2008; Elliott and Barber, 1998.[16]

Type of azotemia	USG	Urine Volume	Cause
Prerenal	≥ 1.035	Decreased	↓ GFR due to dehydration, blood loss, shock or ↓ cardiac output
Renal	< 1.035	Mostly increased	Renal disease
Postrenal	Variable	Decreased	Urinary tract obstruction, uro-abdomen.

1.4.1.4 Proteinuria and systolic blood pressure

Systemic hypertension may cause progression of kidney disease. Glomerular hypertension might develop, and consequently glomerular injury and proteinuria, since cats with CKD cannot compensate for increased glomerular pressure.[12] Hypertension is defined as systolic blood pressure (SBP) > 160 mmHg, and needs to be measured according to the American College of Veterinary Internal Medicine (ACVIM) consensus guidelines using a Doppler ultrasonographic technique.[22] Efforts should be taken to avoid white coat hypertension.[47] Hypertension is not predictive of survival, but is associated with proteinuria, which has been shown to be a major contributor of renal disease progression and mortality.[20,30,48] It is currently unclear if proteinuria itself contributes to progression, or that it is only a marker for renal deterioration.[49] Urine of a healthy cat also contains a small quantity of protein, but the term "proteinuria" as diagnosis means detection of an excessive amount of proteins in the urine.[50] Proteinuria can be classified according to the likely source of proteinuria.[50] It is important to eliminate pre- and postrenal proteinuria and non-renal proteinuria, by following sequential steps.[50] Only persistent renal proteinuria, i.e. proteinuria demonstrated in three or more urine samples over at least a two-week period, is indicative for CKD.[42,50] It is a consequence of increased permeability of the glomerular barrier and impaired reabsorption of the proximal tubules.[51] The severity can be determined by measuring the 24h protein excretion, or UPC, which has been shown to be highly correlated with the 24h protein excretion.[52] In cats, proteinuria is defined as UPC ≥ 0.4 or microalbuminuria in three or more samples collected two or more weeks apart that cannot be attributed to a pre-or post-renal cause.[50] Most cats with CKD only have mild proteinuria (UPC < 1.0), and it has been shown that an increasing UPC, including UPC values within the reference range are associated with an increased mortality both in azotemic and non-azotemic cats.[30,50,53]

The most commonly used proteinuria screening test is the colorimetric Dipstick test, but the outcome is influenced by pH, sediment and concentration of albumin and urine, which may lead to false positive or negative results.[54] Measurement of UPC is always recommended for CKD screening, to evaluate severity of renal lesions. Changes in UPC should always be interpreted together with sCr concentrations, as a decrease in functioning nephrons may cause a decrease in UPC. Improving renal function is reflected in decreasing UPC with stable sCr, whereas decreasing UPC and increasing sCr are suggestive for CKD progression.[54]

Both cats and dogs with CKD are categorized into stages from IRIS to obtain uniform guidelines for diagnosis, treatment and prognosis.[12] Pets are staged over several weeks when they are fasted and well hydrated.[12] Stages 1–4 are based on sCr concentration, and substages are based on UPC and SBP (Figure 8).

1.4.1.5 Medical imaging

Medical imaging, particularly ultrasonography, may help to identify kidney disease and potentially an underlying cause of renal disease.[12] Major ultrasonographic changes include decrease in renal size, increased cortical echogenity, decreased corticomedullary distinction and poorly discernible internal architecture.[55,56] However, normal renal ultrasonography does not eliminate the possibility of renal disease.[32] Further, some abnormalities such as increased cortical echogenity have been described in clinically normal cats,[57] and ultrasonographic findings do not correlate with the degree of renal dysfunction.[56] Therefore, ultrasound is an insensitive and unspecific method for diagnosis of CKD, but essential to find an underlying cause.

Figure 8. IRIS staging of feline CKD. Adapted from www.iriskidney.com (accessed 19/02/2015);[42] Elliott and Watson, 2014.[58]

Stage sCr (µmol/L)	
Stage 1	< 140
Stage 2	140–250
Stage 3	251–440
Stage 4	> 440

Substage proteinuria (UPC)	
Non proteinuric	< 0.2
Borderline proteinuric	0.2–0.4
Proteinuric	≥ 0.4

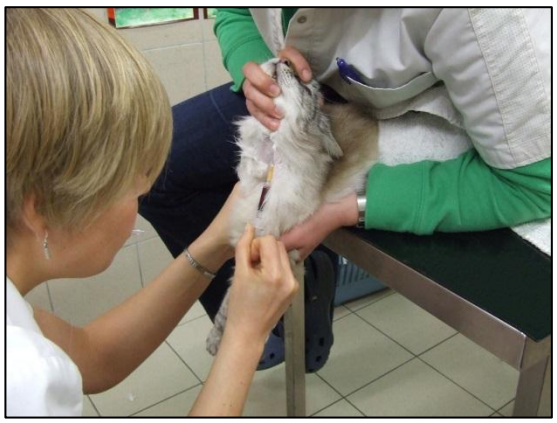
Substage SBP (mmHg)	
Normotension	< 150
Borderline hypertension	150–159
Hypertension	160–179
Severe hypertension	≥ 180

1.4.2 Reference method for kidney function evaluation: GFR

Determination of GFR is the best method to evaluate kidney function.[7] GFR is estimated by the renal clearance of a substance. Renal clearance is the volume of plasma that is cleared of the substance during a given interval of time.[25,59] The renal clearance of a substance equals GFR if the marker is not protein bound, is solely excreted by filtration of the kidneys, is not secreted or reabsorbed by the tubules, is not toxic and does not alter GFR itself.[59] Urinary clearance requires bladder catheterisation, collection of timed urine samples and continuous infusion, which makes this an unpractical method for clinical settings.[60] Plasma clearance methods are more commonly used in cats, with several markers: radiolabelled markers, exogenous Cr and iohexol and inulin. The plasma clearance method involves a bolus dose of the filtration marker with several timed blood samples.[39,61-64] The calculated GFR depends on the used pharmacokinetic model, and the number and timing of blood samples.[59] In practice, these methods are advised for cats with questionable renal function, but with normal renal parameters.[12] Nevertheless, these methods are very time-consuming, for an accurate GFR estimate and therefore not routinely used in practice (Figure 9,10).

Figure 9. Intravenous injection of exogenous Cr and iohexol.

Figure 10. Jugular venepuncture in a combined exogenous Cr-iohexol clearance test.



Therefore, several attempts were made to develop more user- and cat-friendly methods, such as limited sampling methods.[65-70] However, most of these methods were developed in healthy cats. Currently, only two studies have evaluated the limited sampling strategies in a large group of cats with different GFR.[65,69] A limitation of the markers to perform clearance tests is that they require expensive and labour-intensive assays. Iohexol is determined with HPLC, an expensive test. Plasma Cr can be measured more easily, but injectable exogenous Cr is not commercially available. In humans, several equations for GFR-estimation are available based on sCr concentration and demographic variables. The CKD-Epidemiology Collaboration (CKD-EPI) equation has replaced the Modification of Diet in Renal Disease (MDRD) Study Equation in 2009, and is most commonly used.[71] In cats, regression models were developed based on the routine renal variables sCr, serum urea, USG, UPC and SBP, but the low coefficients of determination indicated that these formulas are not able to predict GFR accurately.[69]

1.5 Need for new biomarkers for early diagnosis of feline chronic kidney disease

The main objective in management of CKD is an early therapeutic intervention to slow down disease progression. Cats with CKD often hide symptoms, impeding early diagnosis.[29] Therefore, cats are most often diagnosed in late stages of the disease, making intervention less effective.[29] It has been shown that survival time for cats diagnosed and treated in an early stage is significantly longer than in a more advanced stage.[26] Therefore, it is suspected that early diagnosis will prolong life expectancy and improve quality of life. In humans, screening patient groups at risk for CKD, eg. diabetes or hypertension, is one of the major health tools for the early detection of CKD.[72] It has been shown in humans that early diagnosis prevents important disease complications.[72] However, we have to keep in mind that human CKD is a syndrome, caused by various renal diseases, most of them glomerular diseases. This is in contrast to cats, in which tubulointerstitial nephritis is the most common diagnosis.[29] This difference might be important for screening of cat-groups at risk and for the selection of screening tests. In contrast to humans, diabetic kidney disease seems to be less important in cats.[72] Screening of elderly cats, hyperthyroid cats and breeds predisposed for kidney disease such as Persian and Abyssinian are recommended.[72] In humans, estimated GFR formulas facilitate earlier diagnosis, but those are not yet available for cats. Direct measurement of GFR is considered the best overall index for evaluating kidney function.[7] However, for an accurate GFR-estimation, this procedure is labour-intensive and time-

consuming, making it an inappropriate method for routine use in daily practice.[34] For that reason, the indirect markers of GFR, sCr and urea, are most commonly measured, but they are insensitive and unspecific. It is assumed that these increase only when approximately 75% of kidney function is lost.[20] Especially cats in IRIS stage 1 and early stage 2 are challenging to identify, since their Cr concentration is mostly within the laboratory RI, and other abnormalities i.e. USG < 1.035, renal proteinuria, abnormal diagnostic imaging findings, abnormal kidney biopsy findings, are not always present or not detected by veterinary practioners.[42] Therefore, there is a need for new methods to rapidly and easily detect early feline CKD. In an early stage of the disease, secondary changes are only small, making identification of the underlying cause more plausible. If the underlying cause is treatable, stabilisation or maybe improvement of the kidney function can be achieved, but in most cases, the changes are permanent. In more advanced stages, changes are irreversible, impeding identification of the underlying cause, hindering treatment and deteriorating prognosis.[73] Limited sampling strategies to estimate GFR have been investigated, but are not yet ready for use in practice. To give an accurate GFR estimate, these methods still require different samples, and remain unpleasant and time-consuming. Measurement of a renal marker that only requires one blood or urine sample to evaluate kidney function with high sensitivity, is one of the major objectives in feline nephrology.

Prior to the present thesis, several attempts were made to diagnose CKD in cats early. Several urinary biomarkers may allow localization of the kidney damage. Albumin is a marker for glomerular damage,[51] but is not specific for CKD and does not show advantage over UPC.[49,74] Besides glomerular markers, cats with CKD show a significantly higher urinary concentration of several tubular markers, such as cauxin, retinol binding protein (RBP), N-acetyl-β-D-glucosaminidase (NAG) compared to healthy cats.[75-77] Also the inflammatory cytokines, transforming growth factor-β1 (TGF-β1) and interleukin-8 (IL-8) were significantly higher in cats with CKD compared to healthy cats.[78,79] However, it is currently unknown if these markers allow detection of early feline CKD. Therefore, further research towards early detection of feline kidney damage was warranted.

A possible and promising marker is Cystatin C (CysC). Cystatin C is a low molecular weight (LMW) 13 kilodalton (kDa) protein and proteinase inhibitor involved in the intracellular protein catabolism that is produced at a constant rate, because it is encoded by a housekeeping gene.[80] Studies in rats have shown that there is no plasma protein binding,

which allows glomerular filtration without restriction.[81,82] Cystatin C is reabsorbed in the proximal tubules by megalin-mediated endocytosis and is completely catabolized.[83] It is generally accepted that no tubular secretion of CysC occurs.[82,84] CysC has many properties in humans that are ideal for endogenous GFR marker applications such as: constant production and plasma concentration in the absence of GFR variation, low intra-individual variability, no plasma protein binding, no tubular secretion or reabsorption and no extra-renal clearance.[84] Cystatin C is considered superior to sCr in detecting renal dysfunction in humans.[85] If sCysC has the same advantages in cats as in humans and dogs, this marker can improve early diagnosis of feline CKD, timely treatment, and prolong life expectancy. Furthermore, urinary CysC (uCysC) concentrations are extremely low in healthy individuals compared with individuals with renal tubular damage.[86,87] Therefore, uCysC can be used as a marker for proximal tubular damage. This marker sparked our interest to investigate in cats. However, we have to take into account the between-species difference. A homology of 70% in amino acid sequence between human and feline CysC has been reported.[88,89] It is currently unknown if this difference has consequences for the physiological role of feline CysC and for its evaluation.

2. Cystatin C: a new renal marker and it potential use in small animal medicine

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2.1 History

In the early 1960s, a new protein was discovered in normal human cerebrospinal fluid[90] and the urine of patients with proteinuria.[91] The highest concentration of this protein was measured in cerebrospinal fluid, followed by plasma, saliva and urine,[92] which suggested production in the central nervous system, and catabolism by the kidney.[93-95] The single polypeptide chain contained 120 amino acids, and the molecular mass was 13.260 kDa.[93] Abrahamson et al. observed expression in every examined tissue, including kidney, liver, pancreas, intestine, stomach, lung, placenta, seminal vesicles and salivary gland.[80] Due to similar activity as cystatin A and B, this new protein was named cystatin C.[96] These cystatins inhibit the activity of cysteine proteinases, and therefore protect host tissue against destructive proteolysis.[97]

Cystatin C in serum was investigated as a potential marker for GFR, because a better correlation was observed between the reciprocal of CysC and GFR, compared with the serum concentrations of other measured LMW proteins such as beta-2 microglobulin, retinol-binding protein and factor D.[98,99]

2.2 Assays

2.2.1 Human medicine

In 1994, a fully automated particle-enhanced turbidimetric immuno-assay (PETIA)[a] for CysC was developed and validated in serum[100] and urine.[101] A few years later, a particle-enhanced nephelometric immuno-assay (PENIA)[b] was validated in serum[102] and urine.[103] Concentrations of CysC measured in serum using PENIA showed good correlation with those obtained with the PETIA.[102,104,105] However, this correlation was not observed above concentrations of 2 mg/L, with the PETIA yielding lower concentrations.[106]

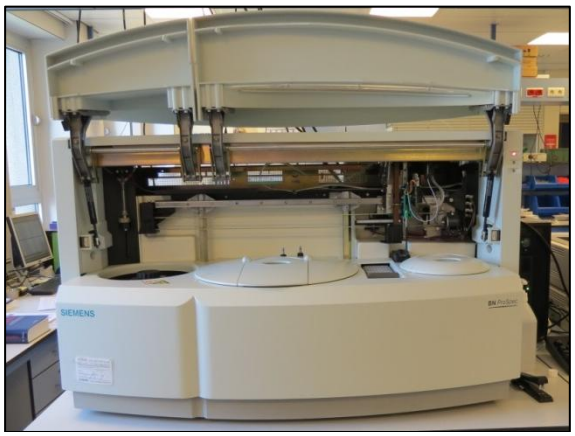


Figure 11. The human particle-enhanced nephelometer (PENIA).

Both turbidimetry and nephelometry are based on the dispersion of light, caused by immune complexes formed by CysC and latex particles coated with polyclonal antibodies. In the turbidimetric assay, the particles are polystyrene particles that are 38 nm in diameter,[100] and in the nephelometric assay, the particles are chloromethylstyrene particles that are 80 nm in diameter.[107] Both assays use polyclonal rabbit anti-human CysC antibodies. The major difference between these 2 methods is that PENIA[b] can only be used with a specialized auto-mated immunonephelometer (Figure 11), whereas PETIA[a] can be used with several analyzers, including the Cobas Fara analyzer[c],[108] Hitachi analyzer[d],[109] Cobas 6000 analyzer[e] [110] and Abbott Architect ci8200[f].[111]

Newer devices are available, but are limited for veterinary use due to high cost. No interferences of triglycerides (≤ 8.5 mmol/L), bilirubin (≤ 150 µmol/L), hemoglobin (≤ 1.2 g/L) or rheumatoid factors (< 3230 kIU/L) were observed for PETIA[a].[100] PENIA[b] showed even less interference.[107]

Similar to Cr, standardization regarding CysC has been accomplished and certified reference material (ERM-DA471/IFCC), is available both for both PENIA[b] and PETIA[g] analyzers and enzyme-amplified single radial immunodiffusion.[112-115]

2.2.2 Small animal medicine

Currently, veterinary assays for measurement of CysC are not available. Therefore, results in animals obtained using the assays designed for humans, do not reflect exact CysC concentrations. An amino acid sequence homology of approximately 70% between human and feline CysC has been reported.[88,89] In dogs, homology between 46% and 79% has been reported;[116] but others have reported a maximum and minimum amino acid sequence homology of 63% and 22% respectively.[89] Cystatin C was first demonstrated in canine amyloid plaques.[117] This finding was of major importance, because the authors demonstrated cross reactivity between the rabbit anti-human CysC antibody from human PETIA[a] and canine CysC present in cerebrospinal fluid. Based on those findings and the studies in humans, Jensen et al.[118] performed the first validation study using PETIA[a] to measure sCysC in dogs (Table 2). Several other authors also have measured sCysC with PETIA[a] in healthy dogs and in dogs with kidney disease.[119-123] Cross reactivity between sCysC and the polyclonal rabbit anti- human CysC antibody by western blotting was only shown in 1 report,[120] and analytical validation parameters were sufficient for PETIA[a].[118,120,123] PETIA[a] also was validated for measurement of canine urinary CysC.[124] Miyagawa et al.[125] also measured canine sCysC with a non-commercially available enzyme-linked immunosorbent assay (ELISA) using the same antibody from PETIA,[a] but this technique is not suitable for everyday practice. Jonkisz et al.[126] observed significantly different results for serum CysC as measured by PENIA[b] among dogs of all IRIS stages, which was not observed with PETIA[a]. Based on those findings, the authors suggested that PENIA[b] is more precise. In our opinion, parallel validation of both PENIA[b] and PETIA,[a] and correlation with GFR measurements are necessary to determine which assay is most appropriate for veterinary use.

Nakata et al.[88] developed recombinant feline CysC in *Escherichia coli* and 3 monoclonal antibodies against this protein. These antibodies were also able to recognize native feline CysC. These authors aimed to design a sensitive and specific sandwich ELISA to detect feline CysC, but this assay is not yet available. Prior to this thesis, no adequate validation of PETIA[a] and PENIA[b] was performed to evaluate feline CysC. This requires further investigation.

Table 2. Validation parameters of human CysC assays in canine veterinary medicine.

Authors	**Assay-analyser**	**Samples**	**Inter-assay CV (%)**
Jensen et al.[118]	PETIA (Cobas Fara II, Hoffman-La Roche, Switzerland)	Low sCysC (< 1.1 mg/L) Medium sCysC (1–2 mg/L) High sCysC (> 2 mg/L)	9.6 5.9 1.7
Almy et al.[120]	PETIA (Hitachi 912, Roche, IN, USA)	Low sCysC Medium sCysC High sCysC	4.7 4.7 2.9
Wehner et al.[123]	PETIA (Hitachi 911, Roche, Germany)	High sCr Normal sCr	2.9 3.6

CysC, cystatin C; CV, coefficient of variation; PETIA; particle enhanced turbidimetric assay; sCr, serum creatinine.

2.3 Cys C and GFR

2.3.1 Human medicine

Several studies in humans have shown that the reciprocal of sCysC correlates more closely with GFR as measured by exogenous clearance tests, than the reciprocal of sCr (Table 3). Additionally, no significant correlation was observed between the reciprocal of sCr and GFR in patients with normal GFR, whereas the correlation with the reciprocal CysC concentration extended to the entire GFR range and remained significant.[100] However, the correlation between GFR and the reciprocal of sCysC is weak in healthy individuals.[127]

Table 3. Correlation data for comparisons between the reciprocal of sCysC or sCr and exogenous marker clearance in humans.

Author	**GFR-marker**	**Correlation coefficient (*r*)**	
		sCysC	**Cr**
Grubb et al.[98]	^{51}Cr-EDTA	0.77	0.75
Simonsen et al.[99]	^{51}Cr-EDTA	0.75	0.73
Kyhse-Andersen[100]	Iohexol	0.87	0.73
Newman et al.[107]	^{51}Cr-EDTA	0.81	0.50
Bökenkamp et al.[128]	Inulin	0.88	0.72
Randers et al.[129]	^{99m}Tc-DTPA	0.87	0.81
Risch et al.[130]	[^{125}I] iodothalamate	0.83	0.67
Stickle et al.[131]	Inulin	0.77 (4−12 years) 0.87 (12−19 years)	0.84 (4−12 years) 0.89 (12−19 years)
Nitta et al.[132]	Inulin	0.84	0.72

sCysC, serum cystatin C; sCr, serum creatinine; GFR, glomerular friltration rate; Cr, creatinine; ^{51}Cr-EDTA, 51-chromium-labeled ethylenediamine tetra-acetic acid; ^{99m}Tc-DTPA, 99-metastabile-technetium-labeled diethylenetriamine penta-acetic acid.

The sensitivity and specificity of the 2 variables were compared by receiver operating curve (ROC) analysis, and sCys C had a higher sensitivity and negative predictive value in detecting a decreased Cr clearance as compared with sCr.[107] Serum CysC concentration began to increase when the GFR decreased, whereas sCr did not change.[107,133]

In human medicine, equation formulas were developed in patients with CKD and are commonly used to estimate GFR based on sCr[71,134-139] or sCysC.[140-143] Equation formulas based on sCysC provided a more accurate and precise GFR estimate than those obtained with the sCr concentration[140] and did not underestimate the measured GFR.[142] However, an equation including both plasma Cr and sCysC provided better results than all the other equations, especially in patients with early stage renal impairment.[144,145]

In humans, sCysC has a larger intra-individual variation and smaller inter-individual variation compared with sCr, which leads to a higher critical difference for the comparison of sequential serum concentrations for CysC.[146] These findings lead to the assumption that sCysC is better as a screening test for decreased GFR, and that sCr is better for monitoring

changes in established renal disease.[147] Serum CysC showed no advantages over sCr in patients with advanced chronic renal insufficiency.[147,148] Additionally, in the general healthy population, GFR equations based on CysC were not superior compared with those based on Cr.[149] Authors have attributed the large bias of GFR equations to the fact that the equations were developed in populations with CKD and low GFR, because non-renal factors may differ between patients with CKD and healthy individuals.[149] Non-renal elimination and lack of CysC measurement standardization may contribute to the observed differences.[147] Therefore, in human medicine, sCysC is used as an additional marker for GFR evaluation, without replacing sCr.

2.3.2 Small animal medicine

Cystatin C has been evaluated as an endogenous indirect marker for GFR in dogs.[120,123,125] Dogs with CKD had significantly higher CysC concentrations compared with healthy dogs[120,121,125] and dogs with various non-renal diseases (immune-mediated, endocrine, dermatologic, cardiologic, neoplastic)[118,119,122,123,150] (Table 4). There was overlap in sCysC concentrations between dogs with non-renal disease and healthy dogs[118,125,150] and between healthy dogs and dogs with CKD.[119,122,125] These results indicate that, currently, sCysC is not a good marker for kidney damage. However, no GFR measurement was performed. Thus, early kidney impairment in healthy dogs or dogs with non-renal diseases cannot be excluded.

Furthermore, very limited information is available for dogs with clinical signs of CKD but without azotemia. In 1 study, plasma CysC was increased in only 1 of 7 dogs that met those criteria.[119] No clearance test was performed in that dog, and thus it is unclear whether or not GFR was decreased.[119] In a remnant kidney model in young adult Beagle dogs, correlation with GFR was better for the reciprocal of CysC (r = 0.79) than sCr (r = 0.54) in the first week after the procedure, when the GFR was lowest (0.50 ± 0.15 mL/min/kg). At 10 weeks after the procedure, when GFR was higher (1.00 ± 0.27 mL/min/kg) but still below the RI (3.50–4.50 mL/min/kg),[120] equal correlation was observed for sCysC and sCr.[120] The authors hypothesized that the equal correlation of sCysC and sCr with increasing GFR was due to a difference in inter- and intra-individual variation. The inter- and intra-individual variation for sCysC and sCr was investigated in the dog by calculating the index of individuality (IoI) determined by the analytical, inter-individual and intra-individual coefficient of variation.[151] For parameters with a low IoI, the repeat test results will be similar to the first result and will

not provide new information.[152] If parameters have a high IoI, the ratio of true positives/false positives will increase.[152] In humans, this explains the higher sensitivity of sCysC (high IoI) in detecting renal impairment, but in dogs, sCysC and sCr showed comparable IoI.[151] However, the authors attributed the difference in IoI of sCysC and sCr between humans and dogs to different storage times, different food, different physical activity index and different breeds, which requires further investigation.[151]

A higher sensitivity of sCysC (76%) than sCr (65%) and comparable specificity (87% for sCysC and 91% for sCr) for detecting decreased GFR (< 3.0 mL/min/kg), as measured by an exogenous Cr clearance test, was observed in dogs by Wehner et al.[123] In this study, dogs with normal GFR (≥ 3 mL/kg/min; n = 23), slightly decreased GFR (2.00–2.99 mL/min/kg; n = 22) and markedly decreased GFR (< 1.99 mL/min/kg; n = 15) were included. Cystatin C and sCr had comparable positive predictive values, but sCysC had higher negative predictive value (69%) compared with Cr (62%) for detecting early CKD.[123] There was a slightly better negative correlation between sCysC (r = -0.630) and exogenous Cr clearance compared with sCr (r = -0.572).[123] There was also a better correlation between sCysC (r = -0.704) and plasma iohexol clearance compared with sCr (r = -0.598). In that study, 88 dogs with CKD and 43 healthy control dogs were included.[125]

In cats, Cys C was evaluated in 2 reports, and contradictory results were observed. Martin et al.[153] concluded that plasma CysC was not a valuable marker for the detection of renal impairment, because only 14 of the 75 cats that had clinical signs of CKD and azotemia had CysC concentrations above the upper reference limit of 4.11 mg/L, which was determined by the authors. However, group allocation in this study did not take into account IRIS guidelines, and the RI was not calculated according to the ASVCP guidelines.[40] Until now, GFR has only been measured in 1 report on feline CysC; Poświatowska-Kaszczyszyn[154] found a significantly better correlation between GFR and sCysC (r = -0.51) than between GFR and sCr (r = -0.46), which is comparable to findings in humans[98-100,107,129-132,155,156] and dogs.[120,123,125] An interesting and common finding in the 2 studies is the overlap in sCysC concentrations between healthy cats and cats with CKD. In addition, sCysC also overlapped between the different IRIS stages in the study of Poświatowska-Kaszczyszyn.[154] In Martin et al,[153] no GFR measurement was performed: thus early kidney impairment in the healthy cats cannot be excluded. In the study of Poświatowska-Kaszczyszyn,[154] GFR was calculated, and GFR was also found to overlap between healthy cats and cats with CKD, potentially

explaining the overlap of sCysC for both groups. However, this study lacked information on USG and used the 1-compartment model for GFR calculation. It is generally accepted that 1-compartment models may overestimate true GFR,[59] which recently was confirmed by Finch et al.[62] Thus, correlation between sCysC and GFR should be further investigated, and a reference interval should be calculated according to the ASVCP guidelines.

Table 4. Overview of studies evaluating the use of sCysC in small animal medicine. Serum CysC (mg/L) was expressed as the mean ± SD, median sCysC or (range).

Species	**Authors**	**Status**	**Age (years)**	***n***	**sCysC**
Dog	Jensen et al.18[118]	Healthy Non renal disease CKD	1-9 0.5-13 0.5-9	17 12 8	1.06 1.62 5.01
Dog	Almy et al.[120]	Healthy CKD	Adult Adult	25 25	1.08 ± 0.16 4.37 ± 1.79
Dog	Braun et al.[119]	Healthy CKD (sCr > 133 µmol/L) Signs of CKD, no azotemia Azotemia, no signs of CKD	0.16-16.5	179 27 7 13	0.60 ± 0.31 (0-8.6) (0.2-1.2) (0-1.2)
Dog	Wehner et al.[123]	Healthy (sCr (55.31–108.5 µmol/L)) Reduced ECPC (< 3 mL/min/kg)	0.25-13 0.5-15	99 15	(0.68-1.6) > 1.6
Dog	Gonul et al.[121]	Healthy CKD	1-9 2-13.5	10 20	1.2 ± 0.42 2.96 ± 1.09
Dog	Miyagawa et al.[125]	Healthy dogs CKD Neoplasia Congestive heart disease		76 88 5 5	0.85 ± 0.15 1.23 ± 0.21 0.93 ± 0.13 0.80 ± 0.12
Cat	Martin et al.[153]	Healthy Signs of CKD and azotemia Signs of CKD, no azotemia Azotemia, no signs of CKD	NS	99 75 35 24	1.60 2.64 1.595 1.74
Cat	Poświatowska-Kaszczyszyn[154]	Healthy CKD - IRIS 1 - IRIS I2 - IRIS II3 - IRIS I4	NS	24 46 16 16 6 8	0.7 ± 0.2 1.3 ± 0.6 1.1± 0.3 1.0 ± 0.5 1.4 ± 0.3 1.25 ± 0.6

ECPC, exogenous creatine plasma clearance; NS, not specified.

2.4 Urinary CysC

2.4.1 Human medicine

Cystatin C is freely filtered through the glomerulus, reabsorbed and catabolized in the tubules, as has been shown in rats.[82,157] With normal renal function, CysC can be found in small quantities in the urine.[92] With proximal tubular damage, uCysC increases.[86,87] Urinary CysC was higher in human patients with renal tubular damage compared with patients with proteinuria without tubular damage and a healthy control group.[87,158] Urinary CysC might be more sensitive than other LMW proteins, such α_1-microglobulin and β_2-microglobulin, because uCysC showed the highest correlation coefficient with sCr.[159] However, it is mandatory to measure total proteinuria, because massive proteinuria has been shown to inhibit tubular reabsorption of CysC in experimentally-induced nephropathies[160] and in children with idiopathic nephropathy,[161] causing higher uCysC concentrations and therefore underestimating tubular function.

2.4.2 Small animal medicine

To the authors' knowledge, only 1 report has validated PETIA[a] for measuring canine uCysC in healthy dogs, dogs with renal impairment and dogs with non-renal disease.[124] The assay was linear and precise and the uCysC/uCr ratio was significantly higher in dogs with renal disease compared with healthy dogs and dogs with non-renal disease. Although the results for uCysC seem promising in dogs, additional studies are required. First, uCysC has not yet been investigated in cats. Second, nor in cats and dogs, uCysC was investigated as a marker of early renal damage. Third, canine and feline CysC are not yet available, and therefore, the accuracy of the method could not be evaluated. Fourth, follow-up to evaluate uCysC/uCr as a prognostic marker was not performed. Additionally, the effect of proteinuria on uCysC concentration was not investigated.

2.5 Biological factors influencing sCysC

2.5.1 Human medicine

2.5.1.1 Age and gender

Because the estimation of renal function by sCr requires adjustment for height and body composition, sCysC was studied as an alternative marker for GFR in children and the elderly. Serum CysC showed diagnostic superiority over sCr as a marker for decreased GFR in the pediatric population,[156] and the CysC-based GFR equation was better than the Schwartz formula,[162-164] except in individuals > 60 years old.[165] The superiority of CysC and more common use of an enzymatic assay instead of the Jaffe method to measure Cr resulted in a new Schwartz formula.[166] Interestingly, several studies have shown that sCysC is high during the first days of life, rapid declined during the first 4 months, and then stays constant beyond the first year of life.[167,168] In contrast sCr falls to a nadir at 4 months, gradually increases to adult concentrations by 15–17 years of age.[169] The decrease of both parameters during the first year can be explained by developing renal function, which causes an increase of GFR. The increase of sCr beyond the first year of life is mainly due to increasing muscle mass and body weight,[169] in contrast with sCysC, which is not correlated with muscle mass.[127,170] In an adult population, increasing age, sex, greater weight, greater height, cigarette smoking and higher C-reactive protein (CRP) concentrations were independently associated with higher sCysC concentration before[171] and adjusting for age, sex and weight of individuals for whom GFR was estimated by a urinary Cr clearance test.[172] The latter indicates that these factors may influence sCysC independent of their effects on renal function. However, others have observed no difference between healthy male and female individuals.[173,174]

Serum CysC concentrations were significantly higher in individuals > 80 years compared with individuals between 65 and 80 years of age, which correspond to the inverse change of the predicted Cr clearance.[175] However, no benefit was found for sCysC compared with sCr in detecting early renal impairment.[176]

2.5.1.2 Inter-individual variation

A larger intra-individual variation has been reported for CysC compared with sCr in healthy individuals and in individuals with impaired kidney function,[130,177,178] and a smaller inter-individual variation has been found.[146] Therefore, some authors propose using sCr as the marker of choice for detecting temporal changes in renal function.[146] A possible explanation for the greater intra-individual variation for CysC is the better ability of CysC to reflect small changes in GFR.[130]

2.5.1.3 Food

Serum CysC was unaffected after intake of a cooked meal, whereas the sCr concentration was significantly higher after eating.[179]

2.5.1.4 Storage

Cystatin C generally is considered a stable protein.[84] Cystatin C was stable in serum for 6 months at -80 °C, and for 7 days at temperatures ranging from 20 °C to -20 °C.[105] Others have reported stability up to 1 month at 2–8 °C[180] but only 1 day at ambient temperature (19–23 °C) and 2 days at 4 °C.[101] No significant differences in sCysC concentrations were observed when comparing concentrations of selected proteins in samples stored at -25 °C for 2 years and 25 years, with samples stored for 1 month.[181]

Urinary CysC was stable at urine pH ≥ 5, at both -20 °C and 4 °C for 7 days and at 20 °C for 48h.[103]

2.5.2 Small animal medicine

Serum Cr concentration in dogs is influenced by breed, age, diet and exercise, which may result in errors in diagnosing CKD.[7] Because sCysC appeared to be a sensitive GFR marker, some authors have investigated the effect of physiological factors on sCysC. Plasma CysC was shown to be lower in adult dogs compared with younger and older dogs and lower in dogs with body weight < 15 kg compared to heavier dogs.[119] In the latter study, 179 dogs were included: 89 young dogs (< 1 year), 39 adult dogs (1–8 years) and 51 old dogs (8–16.2 years). An overlap in plasma CysC concentration was observed (0.12–1.10 mg/L in the adult dogs, 0–1.73 mg/L in the young dogs and 0–1.60 mg/L in the old dogs). Moreover, it was unclear whether all of the dogs were healthy, because complete blood count (CBC), serum

biochemistry or urinalysis were not performed. Other studies did not find a correlation between sCysC and age or weight.[123,151] No circadian rhythm or sex difference were observed.[119,123] In Wehner et al., 99 healthy dogs were included, with an equal gender distribution (52 female, 47 male dogs) and a wide range in age and body weight (3 months–13 years; 5–42 kg).[123] In contrast, the study of Pagitz was limited by including only 24 healthy dogs (16 female and 8 male) with an age range of 10–97 months.[151] Because contradicting results were reported regarding the effect of age and bodyweight on sCysC in dogs, additional studies in a larger number of healthy dogs, preferably in which GFR is measured, are required.

In contrast to plasma Cr concentration, which increases in dogs during the first 12 h after a meal, plasma CysC concentration showed a dramatic decrease during the first hour after a meal. This decrease lasted for 9 h, and then returned to baseline after 12 h.[119] Based on these results, dogs should be fasted for at least 12 h before taking blood samples to measure CysC concentration. Creatinine originates primarly from the amino acids glycine, arginine and methionine, but also from the gastrointestinal tract, which can explain the increase after a meal.[182] Because plasma CysC concentration is mainly determined by GFR, and it has been shown that a meal causes a significant increase in GFR,[183] the increased clearance of CysC could explain the decreased concentration, but this has not yet been confirmed.

To the authors' knowledge, no studies about the biological variation of sCysC in cats have been performed.

2.6 Clinical factors influencing sCysC

2.6.1 Human medicine

2.6.1.1 CysC in patients with diabetes mellitus

Diabetic nephropathy is a common complication in human diabetes patients and is characterized by persistent albuminuria and an associated decrease in GFR.[184] Several studies have reported that sCysC is a better GFR marker than sCr for the early detection of incipient diabetic nephropathy.[185,186] Moreover, the correlation between GFR measured with ^{51}Cr-EDTA and sCysC (r = 0.84) was significantly stronger compared using estimated GFR (r = 0.70).[186] However, others have reported that sCysC is equal to sCr as a GFR marker in micro-

and macro-proteinuric diabetes patients.[187] This difference can be explained by the different methods used to measure sCr, differing GFR reference methods and varying diabetes populations studied.

2.6.1.2 CysC and AKI

Acute kidney injury (AKI) is associated with high mortality. Therefore, early detection is critical to prevent further progression.[188] Serum CysC concentration could detect development of AKI 1 or 2 days earlier than sCr concentration in intensive care patients with ≥ 2 predisposing factors of AKI.[188] A limitation of this study was that GFR was not measured. Interestingly, the uCysC concentration also may predict renal replacement requirement in patients initially diagnosed with non-oliguric acute tubular necrosis.[189] In similar studies, CysC was as effective as[190] or less sensitive than[191] sCr in the detection of AKI. However, similar to sCr, CysC could not discriminate between CKD and AKI.[192] In conclusion, several authors[193,194] have suggested that the use of CysC to detect AKI must be evaluated in larger studies and with different types of AKI and that the prognostic value also must be determined.

2.6.1.3 CysC and thyroid function

In patients with hyperthyroidism, renal blood flow is stimulated, which causes increased GFR.[195] Serum Cr concentration decreases, which masks patients with concurrent CKD.[196] Contrasting effects have been observed in patients with hypothyroidism.[197,198] As sCysC was introduced as a new marker of kidney function, the impact of thyroid dysfunction on sCysC also was investigated. With therapy, sCysC concentration increased in patients with hypothyroidism, and decreased in patients with hyperthyroidism.[199-201] However, others did not observe higher or lower sCysC concentrations in patients with untreated hyper- or hypo-thyroidism respectively.[202] When considering sCysC concentrations in patients with hyperthyroidism, GFR is overestimated and, in patients with hypothyroidism, GFR is underestimated.[203] Den Hollander suggested that there is an increased or decreased production of CysC in hyper- and hypothyroidism respectively, due to the influence of the thyroid state on general metabolism.[204] Serum concentrations of CysC and TGF-β1 were significantly higher in patients with hyperthyroidism and a positive correlation between sCysC, thyroid hormones and TGF-β1 was observed.[205] After treatment, sCysC and TGF-β1 decreased. In vitro findings have suggested an increase in TGF-β1 concentration in hyperthyroidism and a stimulatory effect of thyroid hormones and TGF-β1 on CysC production.[205]

2.6.1.4 CysC and cardiovascular risk

Chronic kidney disease is a known risk factor for ischemic heart disease. In contrast with sCr, CysC was associated with an increased risk of heart failure.[206] Serum CysC tends to be a stronger predictor of mortality than sCr in elderly individuals with heart failure,[207] as well as in the entire elderly population.[208] Because CysC is a proteinase inhibitor that plays an important role in tissue remodelling, the higher CysC concentration also could represent a compensatory mechanism in vascular injury.[209]

2.6.1.5 CysC and cancer

Because renal disease has a high prevalence in the elderly, concurrent neoplasia may be present. Decreased regulation by cystatins is responsible for increased cysteine protease activity in tumor cells.[210-212] Cystatin C has two anti-tumor effects. First, it is a major inhibitor of the cathepsins, enzymes that cause degradation of basal membranes by tumor cells. Therefore, CysC suppresses the metastastic process.[84] Second, CysC also inhibits TGF-β and its TGF-β signaling pathway.[213,214] The specific role of CysC in oncogenesis has not yet been elucidated. However, individuals with untreated carcinomas[215] and leukemia[216] had significantly higher sCysC concentrations compared with patients after therapy. However, two other studies[155,217] did not find a difference in sCysC concentrations between patients with malignancy and a healthy control group.

2.6.1.6 CysC and inflammation

In vitro, CysC regulates certain aspects of immune function,[218] because IL-10 controls CysC synthesis in response to inflammation.[219] Several reports have shown a good correlation between sCysC and other inflammatory markers,[172,220,221] but these studies were performed in populations with either cardiovascular[221] or renal impairment,[220] which can cause bias. Dexamethasone caused a dose-dependent increase of CysC secretion in vitro,[222] in vivo, sCysC is influenced by prednisolone administration.[223,224]

2.6.2 Small animal medicine

One study in dogs showed no influence of inflammation on sCysC.[123] However, only a limited number of dogs was examined; therefore additional research is needed to examine the impact of this condition on sCysC. Because glucocorticoids are commonly administered to small animals, future studies are needed to evaluate whether corticosteroids falsely increase sCysC. Serum CysC concentration and GFR should be measured in healthy dogs and cats before, during and after glucocorticoid administration.

In a study comprising 10 volume-depleted dogs, and one dog with AKI, a weaker correlation between sCysC and GFR than sCr and GFR was observed.[120] These results indicate that CysC is not a good GFR marker for decreased GFR due to pre-renal causes. However, caution is warranted. Only a few dogs were sampled, which could have influenced the regression analysis. Furosemide administration used to achieve volume depletion also could have affected CysC kinetics.[120] In the same study, the sCysC concentrations of the dog with AKI fell within the RI established for healthy dogs,[120] in contrast to observations in human patients.[133] This suggests that in dogs with AKI, sCysC is not a sensitive indicator of decreased GFR. However, in critically ill dogs, sCysC concentrations were significantly higher in dogs in shock compared with healthy dogs,[225] but this result was not observed in multiple-trauma dogs, which is in contrast to reports in human.[226] To date, no large-scale study in dogs with AKI has been performed to evaluate sCysC.

One of the diseases leading to AKI in dogs is babesiosis, and the diagnosis of this serious complication is difficult. Photochemistry assays can cause false-positive results in babesiosis due to free haemoglobin or bilirubin.[227] In one study, no increased sCysC or sCr concentration was observed. Studies investigating correlations between GFR and sCr and sCysC should be performed to identify the most appropriate marker for screening for renal damage in dogs with babesiosis.[227] In our opinion, additional studies in dogs with AKI or pre-renal azotemia are needed.

Cystatin C also was of particular interest in dogs with visceral leishmaniasis, a disease that results in CKD due to immune complex dispositions and glomerular injury.[228] In humans, sCysC concentrations were positively correlated with circulating immune complexes and the production of granulocyte-macrophage colony stimulating factor (GM-CSF), 2 factors leading

to glomerular dysfunction in leishmaniasis.[229] In dogs with visceral leishmaniasis, mean sCysC concentration was significantly higher than in the control groups, and sCr concentration was lower than in the control group, although not significantly. However, the mean sCysC concentration fell within the RI proposed by two other authors.[119,150] GFR should be determined and renal biopsies should be performed to determine if the increased sCysC concentration in dogs with leishmaniasis is due to immune-complex deposition or an extra-renal factor.

In human medicine, contradictory reports have been published regarding the effect of different tumors on sCysC concentration. Therefore, studies in small animals to evaluating the effect of neoplasia on sCysC are essential. Cystatin C is an anti-tumor marker, because it is a protease inhibitor, and therefore it inhibits damage from the tumor cells and the metastatic process. Serum Cr concentration is not a good GFR marker in patients with neoplasia due to the decreased muscle mass, and sCysC potentially may be a valuable alternative.

Cystatin C has not yet been investigated in cats with non-renal disease, except for hyperthyroidism. Serum CysC was evaluated in cats with hyperthyroidism using PETIA[a]. No correlation was observed between GFR measured by exogenous inulin clearance and 1/sCysC concentration, although a significant correlation between GFR and 1/sCr was observed.[230] Additionally, no significant decrease in sCysC concentration was observed after treatment with ^{131}I.[230] Although preliminary, the study of Jepson et al.[230] suggests a potentially similar influence of thyroid function in cats as in humans, with hyper- and hypothyroidism causing increased or decreased sCysC concentrations, respectively. Additional studies to clarify the impact of thyroid function on CysC are warranted.

3. Validation of biomarkers

Before CysC can be used in small animal practice, this marker needs to be validated. Validation is defined as the evaluation of the quality of a measurement, in order to identify the presence of an analyte, and to evaluate the status of the test subject.[231] It is fundamental to investigate if the measured concentration of the marker reliably reflects the status of the animal.[232] Hence, validation is composed of analytical, biological and clinical aspects.

3.1 Analytical validation

In practice, validation is performed by evaluating the analytical performance followed by observing how the test behaves in patients.[232] First, the *analytical validation* is determined. It determines the fitness of an assay, that has been developed and standardized for an intended purpose.[233] As described above, no veterinary CysC assays are available. Therefore, in this PhD thesis, it will be tested if the human assays can reliably measure feline CysC. This can be evaluated by determining the imprecision, inaccuracy and analytical sensitivity.[232] The *imprecision* is the level of difference between results of replicates of a sample within and between runs of the same test method in a given laboratory.[233] The within- and between-run imprecision is calculated from the results of replicate measurements on the same specimen on the same day and on several consecutive days respectively.[232] Samples with low, middle and high concentrations should be included. The imprecision is reported as coefficient of variation (CV), and should not exceed 15%.[234] The *inaccuracy* can be defined as the difference between the mean value of a series of measurements and the true value. Inaccuracy can be evaluated by spiking recovery, linearity check, control material or comparison of analytical methods. If the analyte is available, spiking recovery can be performed, by adding a known amount of the analyte in both patient samples and diluent. If the recovery of the spike with samples equals recovery of the spike with diluent, the sample matrix is valid.[232,235] Another method used when the analyte is not available, is linearity check. The analyte concentration is measured before and after dilution with the appropriate diluent. A correlation coefficient of 1 indicates a 100% recovery.[232] Figure 12 illustrates imprecision and inaccuracy. The analytical sensitivity is determined by the limit of detection (LOD), the smallest amount of the analyte in the sample that can be detected. In practice, a sample with low or no analyte concentration is measured several times and the estimated detection limit is calculated based on the mean value and standard deviation.[232,235]

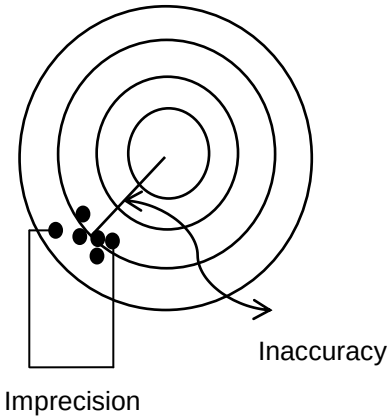


Figure 12. Inaccuracy and imprecision. The true value is in the centre. Imprecision represents the closeness of the repeated measures, whereas inaccuracy is the difference between the true value and the mean of the repeated measures. Adapted from Jensen AL, 2000.[232]

3.2 Biological validation

A second step in analyte validation is to know test results in healthy animals i.e. the *biological validation.*[232] The analyte concentration can be affected by both non-host and inherent host-factors.[231] Non-host factors are technical factors such as contamination or detoriation of the sample, which are essential to be studied, in order to determine appropriate handling of samples.[231] Host-factors can be subdivided in the inherent factors age, sex, breed, nutritional status; and the acquired factors, caused by disease.[231] To know if a measured value of the analyte is abnormal, it is necessary to know an estimated value from a healthy population of comparable individuals, the reference population.[40] A RI is determined in a minimum of 120 reference individuals by a nonparametric method with 90% confidence intervals.[40] It is essential to know the influence of inherent and pre-analytical factors to determine if separate RIs are necessary.[40] Inclusion and exclusion criteria, based on narrowly or extensive defined procedures to define health, should be established.[40]

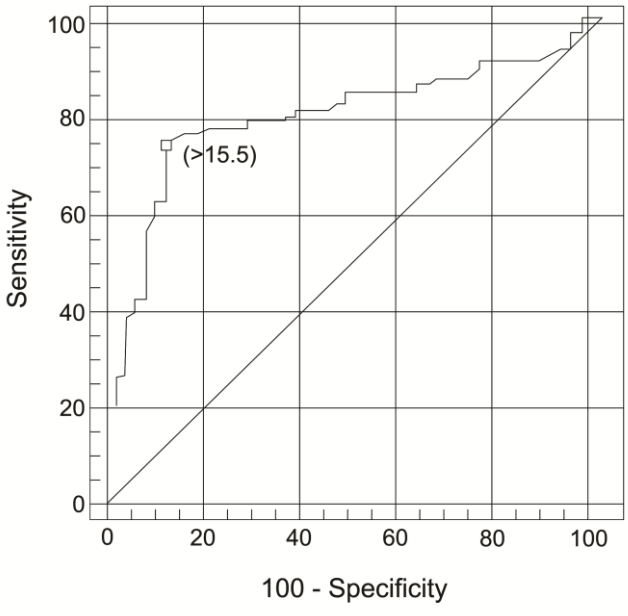
3.3 Clinical validation

A third step is to compare test results in healthy animals with various groups of diseased animals i.e. the *clinical validation.*[232] The ability to discriminate between patients with and without the disease is a major determinant of the clinical utility.[40] To test this, three criteria should be kept in mind: the appropriate animals should be selected i.e. animals for which the disease is a differential diagnosis; the animals should be classified as with or without the disease, independently from the evaluated laboratory test; the test should be performed in batch i.e. at the same point in time for all animals.[232] The result indicates if the

analyte is significantly different between healthy and diseased animals. Next step is to establish a decision threshold, which is selected for characterizing a test results as positive or negative.[40] This can be derived from the result of a gold standard method,[231] or from prospective studies with thorough deliberation and planning.[40] Based on this limit, test results can be classified into positives and negatives and the sensitivity and specificity of a test can be calculated. The sensitivity is defined as the proportion of patients with the disease that test positive and specificity is defined as the proportion of patients without the disease that test negative.[232] The higher the sensitivity and specificity, the better the test. Depending on the chosen cut-off value, dividing test results in negative or positive, sensitivity and specificity change. This can be displayed in a graph i.e. ROC, with sensitivity on x-axis and (1-specificity) on the y-axis.[232] Based on this curve, the overall diagnostic accuracy of the test can be determined visually and mathematically. The better the test discriminates between healthy and disease, the closer the ROC curve is situated in the upper left-hand corner of the graph.[232]

After the validation process, all factors are known that have to be taken into account to criticize the marker.

Figure 13. ROC curve. The sensitivity is plotted as a function of (1-specificity) over the range of selected cut-off values. Tests with a poor performance tend toward the diagonal line, tests with a good performance tend upward and leftward. The highest sensitivity and specificity is the top-leftmost point. In this example, the cut-off value that gives this point is 15.5. Adapted from Jensen AL, 2000.[232]



4. Conclusion

Since routine renal markers, sCr and urea, are insensitive markers of renal function, there is a need for markers that can reliably and easily detect early kidney dysfunction in cats.

Cystatin C has the potential to become a valuable biomarker in small animal medicine, but adequate analytical, biological and clinical validation is needed first.

A few studies using canine serum have been performed, but studies in cats are scarce. This thesis aimed to validate CysC in cats at analytical, biological and clinical level. First, there is a need to perform a thorough analytical validation of the nephelometric and turbidimetric assays for determining CysC in feline serum and urine. These studies will identify which assay is most suitable for feline CysC measurement. To evaluate whether sCysC is a better GFR marker than sCr, it is necessary to evaluate the biological factors that may influence sCysC and to establish a RI. In addition, the correlations of GFR with sCysC and sCr must be compared. To use sCysC as GFR marker in practice, conditions that might influence CysC also must be investigated. Finally, further investigations of uCysC should be performed to assess its value for the detection of tubular dysfunction.

End Notes

[a] PETIA Cystatin C assay, Dako, Glostrup, Denmark

[b] PENIA Cystatin C assay, Siemens, Marburg, Germany

[c] Cobas Fara analyser, Roche Diagnostics, Basel, Switzerland

[d] Hitachi analyser, Roche Diagnostics, Indianapolis, IN

[e] Cobas 6000 analyser, Roche Diagnostics, Basel, Switzerland

[f] Abbott Architect ci8200 analyser, Abbott Laboratories, Abbott Park, IL

[g] PETIA Cystatin C assay, Gentian AS, Moss, Norway

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CHAPTER II

SCIENTIFIC AIMS

Chronic kidney disease (CKD) has a high prevalence in cats. It is a progressive and irreversible disease. Early detection and consequent treatment are essential and may increase median survival time by preventing further renal damage.

The best overall index for evaluating kidney function, is measurement of glomerular filtration rate (GFR), but this procedure is time-consuming and labour-intensive and is not an appropriate method for routine use. The indirect markers, serum creatinine (sCr) and serum urea, both lack sensitivity and specificity. They only increase when up to 75% of the functional renal mass is lost, and they are influenced by non-renal factors.

There is a need for renal markers that can detect feline CKD early and easily. A possible marker is cystatin C (CysC). Studies in humans and dogs revealed promising results, but data in cats are scarce.

The scientific aims of this thesis were:

1. Evaluate precision, accuracy and analytical sensitivity of a commercial available human particle enhanced nephelometric assay (PENIA) and particle enhanced turbidimetric assay (PETIA) for feline CysC determination in both serum and urine.
2. Determine optimal storage conditions for sCysC and urinary CysC (uCysC) and evaluate the effect of feeding on feline sCysC.
3. Determine the effect of age, breed and sex on feline serum CysC (sCysC) and establish a reference interval.
4. Evaluate sCysC and uCysC in cats with hyperthyroidism and cats with feline immunodeficiency virus (FIV)-infection.
5. Evaluate CysC as a marker for CKD in cats.

CHAPTER III

ANALYTICAL VALIDATION OF FELINE CYSTATIN C

ANALYTICAL VALIDATION OF THE HUMAN PARTICLE-ENHANCED NEPHELOMETER AND TURBIDIMETER FOR CYSTATIN C MEASUREMENT IN FELINE SERUM AND URINE

Liesbeth F.E. Ghys[1], Evelyne Meyer[2], Dominique Paepe[1], Joris R. Delanghe[3], Sylvie Daminet[1]

[1] Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

[2] Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

[3] Department of Clinical Chemistry, Microbiology and Immunology, Faculty of Health Medicine and Life Sciences, Ghent University, De Pintelaan 185, B-9000 Ghent, Belgium

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Summary

In people and dogs, Cystatin C (CysC) seems superior to serum creatinine (sCr) to estimate the glomerular filtration rate (GFR). A particle-enhanced nephelometric immunoassay (PENIA) and turbidimeter (PETIA) are available to measure CysC in humans, but there are no reports in cats. The objective of this study was the validation of the human CysC PENIA and PETIA with feline serum and urine, and to perform a pilot study comparing serum and urine CysC between healthy cats and cats with chronic kidney disease (CKD). Protein purification was performed to produce feline recombinant CysC.

The feline CysC gene (CST3) was isolated from white blood cells and the plasmid with CST3 was cloned into 3 different expression vectors: *Escherichia coli, Pichia pastoris* and HEK293T mammalian cells. Western blot analysis was used to assess cross-reactivity between feline CysC and the polyclonal rabbit anti-human CysC antibody from PENIA and PETIA. Imprecision and linearity were determined for feline serum and urine CysC. Serum and urine CysC were measured in 10 healthy and 10 CKD cats.

Feline recombinant CysC could not be produced. Cross-reactivity between the polyclonal rabbit anti-human CysC antibody from both PENIA and PETIA and feline CysC was demonstrated. Intra-assay coefficients of variation for serum and urine were 1.3% and 0.4% for PENIA and 0.8% and 0.4% for PETIA and the inter-assay coefficients of variation 12.5% and 4.1% for PENIA and 9.5% and 15.9% for PETIA. Measured with PENIA, cats with CKD had a significantly higher serum CysC concentration (1.24 (0.63–2.99) vs 0.79 (0.43–1.05) mg/L; $P = 0.02$) and urine CysC/urinary Creatinine (uCr) ratio (565.6 (0–1311) vs < 0.049/uCr mg/mol; $P = 0.005$) compared with healthy cats.

The human nephelometric assay showed satisfactory validation results for feline CysC, in contrast with PETIA. Cats with CKD had a significantly higher sCysC concentration and uCysC/uCr ratio compared with healthy cats. Additional studies are necessary to evaluate CysC as an early marker of renal damage in cats.

Introduction

Chronic kidney disease (CKD) is a common disease in cats and has an estimated prevalence between 1.6% and 20% in the general cat population,[1] and of 30% in cats older than 15 years.[2] Early detection and treatment of CKD are very important to slow down progression of renal disease and improve the long-term prognosis, including the quality of life.[3]

Evaluation of the glomerular filtration rate (GFR) – the volume of ultrafiltrate produced per minute of time – is considered the gold standard to evaluate kidney function.[4] Direct measurement of GFR in animals is based on the clearance of markers that are freely filtered through the glomerulus, and that undergo minimal tubular absorption or secretion.[5] Because accurate determination of GFR is labor-intensive and time-consuming, it is not routinely used in veterinary practice. The routine indirect diagnostic markers for GFR, serum urea and serum creatinine (sCr), are only increased when > 75% of the functional renal mass is lost.[4] This implies that CKD is currently diagnosed in a relatively late stage. Moreover, serum urea and sCr are influenced by food intake, medication and muscle mass.[4] Consequently, there is an urgent need for indirect markers that can reliably and easily detect early kidney dysfunction in cats.

Cystatin C (CysC) is a 13 kDa protein, first described in cerebrospinal fluid of healthy humans.[6] It is part of a family of proteinase inhibitor proteins, which play a major role in the intracellular catabolization of peptides and proteins.[7] It is produced by all nucleated cells at a constant rate, since it is encoded by a housekeeping gene.[8] Most of the properties that are required for an ideal endogenous GFR marker apply for CysC, as shown in the rat.[9] Besides a constant production and constant plasma concentration in the absence of GFR-variation, it shows a low intra-individual variability, exhibits no plasma protein binding, no tubular secretion or absorption and no extra-renal clearance.[10] In addition, as CysC is completely catabolized in the tubulus, only small amounts can be found in the urine of healthy people.[11] In people[12,13] and dogs[14] with tubular dysfunction, urinary CysC (uCysC) concentration is significantly higher compared to that in healthy individuals. This suggests that uCysC can also be used as a tubular marker in people and dogs.

Several studies in people confirm the superiority of serum CysC (sCysC) to sCr in the detection of renal impairment.[15,16] Additionally, in dogs, sCysC has a higher sensitivity and negative predictive value compared with sCr for detecting renal impairment.[17-19] Serum CysC has also been investigated in human patients with risk of acute kidney injury (AKI), one of the major complications of CKD. Serum CysC was able to detect development of AKI one or two days earlier than sCr,[20] but the significantly higher mean sCysC only lasted for 24h.[21]

Three methods for measurement of CysC are available in humans: an enzyme-linked immunosorbent assay (ELISA), a particle-enhanced turbidimetric immunoassay (PETIA) and a particle-enhanced nephelometric immunoassay (PENIA).[22-24] Currently, no commercial veterinary CysC assays are available. Therefore, the human assays need to be validated for the different species of interest. As the immunoassays are based on an antibody-antigen reaction, demonstration of cross reactivity between the feline CysC antigen and the anti-human CysC antibody used in the nephelometric and turbidimetric assay had to be established by Western blotting. The nephelometric and turbidmetric assay are based on dispersion of light by immunocomplexes present in the sample. When reagent is added to serum, CysC will bind antibodies coated with chloromethylstyrene particles (PENIA) or polystyrene particles (PETIA). These aggregates cause dispersion of light and an electric signal. Concentration is determined with a standard curve, based on pure human CysC. This makes that the measured feline CysC concentration is not an exact concentration but relative. Therefore, a feline CysC standard should be produced. Production of feline recombinant CysC has been described earlier.[25]

The first aim of this study was the production of feline recombinant CysC. The second aim was the validation of the human PENIA and PETIA for determination of CysC in serum and urine of cats. After evaluation of the validation parameters, the most satisfactory assay to measure feline CysC was selected. In addition, a pilot study was performed to evaluate if the CysC concentration determined in feline serum and urine significantly differed between healthy cats and CKD cats.

Materials and methods

Study design

For the production of feline recombinant CysC, the feline CysC gene (CST3) was cloned into a plasmid. Consequently, CysC was cloned into an expression vector with a fusion protein, followed by protein purification. For the validation, blood and urine samples were collected from 10 healthy cats and 10 cats with CKD. In all cats, complete blood cell count (CBC), serum biochemistry profile and complete urinalysis were performed. Blood and urine samples of three healthy cats and four CKD cats were selected for Western blot analysis for PENIA and two CKD cats (one proteinuric and one non-proteinuric) and one healthy cat for PETIA. Blood and urine samples of six healthy cats and six cats with CKD were used for assay validation. Serum and urine samples of all 20 cats were analysed with the best assay to measure their CysC concentration. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Local Ethical and Deontological Committee of the Faculty of Veterinary Medicine, Ghent University (EC2011_197). All owners whose animal participated in the study signed an informed consent.

Animals and samples

Adult healthy and CKD cats were included, regardless of breed and sex. Cats were considered healthy when there was no disease history and no abnormalities were detected on physical examination, CBC, serum biochemistry profile and urinalysis. Criteria for normal urinalysis were: urine specific gravity (USG) > 1.035, inactive urinary sediment, urinary protein to creatinine ratio (UPC) < 0.4 and negative bacteriological urine culture.[2] Cats with sCr > 141.2 µmol/L and USG < 1.035 were excluded.

Diagnosis of CKD was made prior to inclusion. Cats were diagnosed with CKD based on the presence of clinical signs compatible with CKD and laboratory (i.e. renal azotemia and USG < 1.035) findings. Cats were classified into four stages according to the International Renal Interest Society (IRIS) guidelines.[2] Cats with evidence of severe concurrent systemic diseases based on their history, physical examination, CBC, serum biochemistry profile and urinalysis were excluded. Likewise, cats receiving medication within one month prior to inclusion, such as nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids,

antibiotics, β-blocking agents, and angiotensin-converting enzyme (ACE), were also excluded.

Analytical Methods

Sample collection

Five mL of blood were collected by jugular venipuncture using a 23 G needle. After centrifugation (5 minutes at 2431 x *g*), one mL of serum was analysed on the same day. The CBC[a] and serum biochemistry profile[b] were performed in all cats. The assay for creatinine was a kinetic Jaffe reaction.[26] A modified totally enzymatic procedure, first described by Talke and Schubert[27] was used to measure serum urea. In cats older than 6 years, serum total thyroxine was also measured. The remaining serum was divided into aliquots of 250 µL and stored at -72 °C until analysis. In one healthy cat, 4 mL of blood were taken on EDTA for CST3 cloning.

Ten mL of urine were taken by cystocentesis with a 22 G needle. The USG was determined using a manual refractometer. Urinalysis consisted of a urinary dipstick test,[c] measurement of UPC,[d] microscopic sediment analysis and bacterial culture.[e] All analyses were performed at a constant room temperature of approximately 20 °C. Urine was centrifuged (3 minutes at 447 x *g*) in a tube, and the urinary sediment was analysed within 30 min. The sediment was prepared by decanting the supernatant, to leave an equal amount of urine and sediment in the conical bottom of the tube. A drop of resuspended sediment in urine was placed on a glass slide and covered with a coverslip. Crystals and casts were evaluated per low-power field (10x objective) and cells were evaluated per high-power field (40x objective).[28] The supernatant and remaining serum were divided into aliquots of 250–300 µL, and stored at -72 °C until analysis (minimum 4 weeks, maximum 76 weeks).

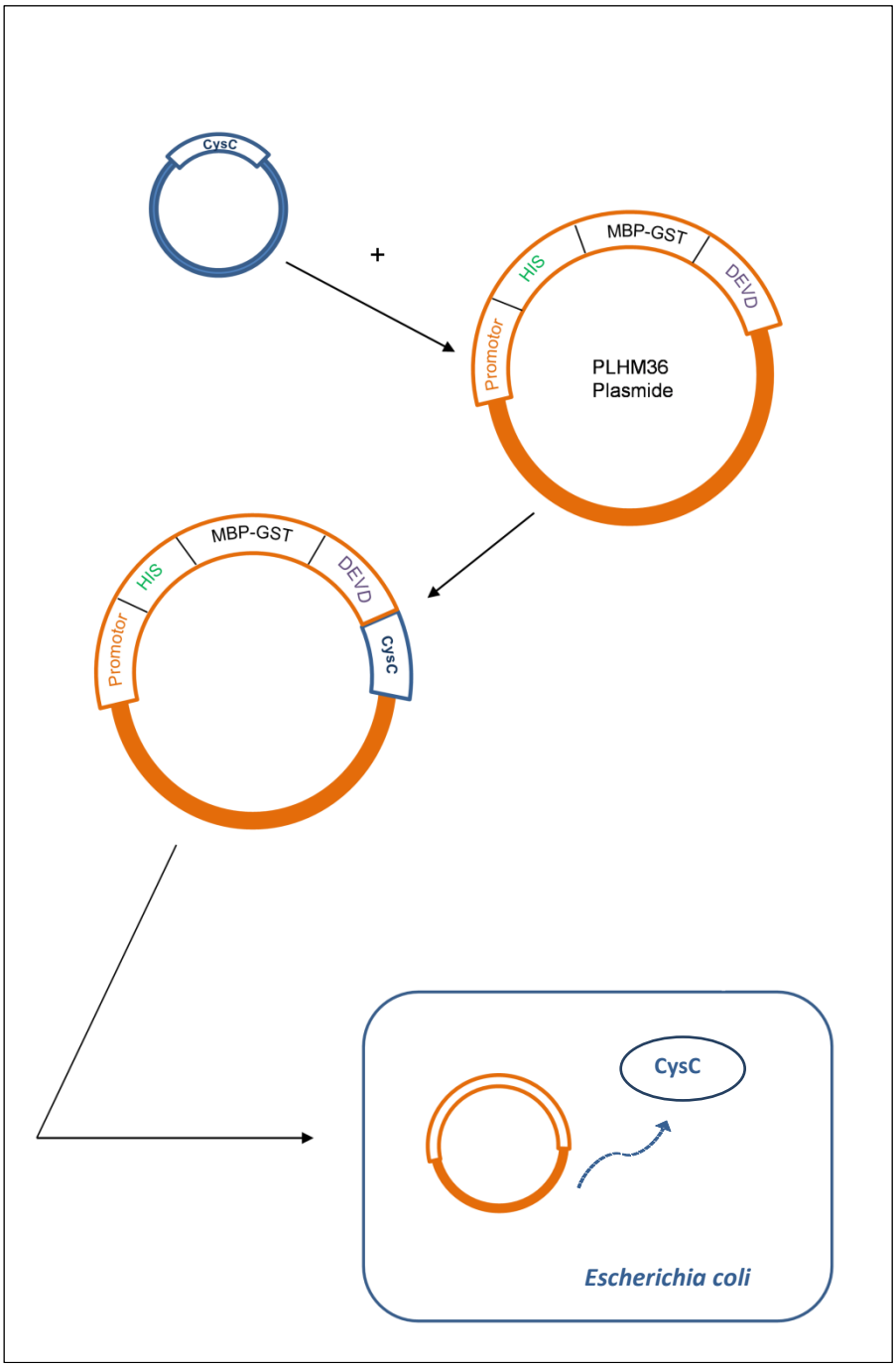
Cloning of CST3 and protein purification

RNA was isolated from the white blood cells with Total RNA Isolation Reagens (TRIR).[f] The quality and quantity of RNA was evaluated by agarose gel electrophoresis and a spectrophotometer.[g] One µg RNA was transformed into cDNA[h] with oligo-dTs and hexameres. The complete coding sequence of feline CST3 was amplified with polymerase chain reaction (PCR)[i] with cDNA as template and primers based on the feline CST3 sequence from the sequence database (Acc.No. NM_001184972). The obtained RT-PCR product of 589 base pairs (bp) was visualized with agarose gel electrophoresis and eluted with a commercial kit.[j] The elution product was ligated into the pCR-II vector and transformed in DH5a competent cells.[k] Six clones were cultivated in 5 mL buffer (with ampicillin as antibiotic), followed by extraction of the plasmid DNA into 50 µL (Birnboim method) with a production of ± 4 µg/µL measured with the spectrophotometer.[g] Clone verification was performed with EcoRI restriction digest analysis on 1µg plasmid DNA. The insert in the pCR II vector was cut, and the size was analysed by agarose gel electrophoresis. The clones with correct insert dimensions were selected, and the insert was sequenced with a commercial kit[l] and the DNA was analysed.[m] Finally, the sequences were analysed with BioEdit.[n]

The plasmid with the feline CysC gene was cloned into expression vectors for *Escherichia coli*, *Pichia pastoris* and HEK293T mammalian cells with and without fusion proteins (maltose binding protein (MBP) glutathione S-transferase (GST) or human serum albumin (HSA)). The fusion proteins enhance solubility and expression of feline CST3. Between CysC and the fusion protein at the N-terminus, DEVD or DELD was inserted, a recognition site for murine caspase-3, to remove the fusion protein during purification. At the N-terminus of the construct (just before the fusion protein), a His-6 Tag was inserted, necessary for the purification. For *Escherichia coli*, two constructs were cloned to test expression: pLHM36CystatinC (MHHHHHH-MBP-DEVD-CystatinC and pLHG36CystatinC (MHHHHHH-GST-DEVD-CystatinC). For *Pichia pastoris* the constructs were: pAoXZalfaH-DEVD-CystatinC and pAOXZalfaH-HSA-DEVD-CystatinC. The construct for the human cells was pCAGGSsH-C-CystatinC (AHHHHHH-MBP-DELD-CystatinC). DEVD or DELD are the recognition sites for murine caspase-3, which cuts CysC immediately after DEVD or DELD. The first amino acid after DEVD or DELD is the first amino acid of CysC. The expressed fusion protein with CysC was purified by Ni-affinity chromatography, followed by murine caspase-3, to remove CysC from the fusion protein. Feline CysC was

finally purified by gel filtration chromatography. A schematic overview of expression in *E.Coli* is shown in Figure 1.

Figure 1. Overview of cloning of CST3 (feline CysC gene) in E.Coli. The plasmid with CysC and the PLHM36 plasmid were cloned into *Escherichia coli* with MBP-GST maltose binding protein or glutathione S-transferase, fusion proteins, enhancing solubility and expression level; His-6 tag, essential for protein purification; DEVD, recognition site for murine caspase-3, cutting the protein right before CysC.



Western blot analysis

Sample volumes of 15 µL serum and 15 µL urine were loaded onto a 12% SDS-PAGE gel,[o] together with a standard[p] and a human CysC standard.[q,r] After gel electrophoresis, the separated proteins were blotted onto a nitrocellulose membrane[s] with a transfer buffer[s] containing 10% methanol. The residual binding sites were blocked for 1 hour at room temperature with Tris buffered saline containing 1% Tween-20 (TBS-T)[s] and 5% milk powder. The membrane was then incubated overnight at 2−8°C with a polyclonal anti-human rabbit CysC antibody[t] at a dilution 1/2000. After washing with TBS-T containing 5% milk powder, the blots were incubated for 1 hour at room temperature with donkey anti-rabbit IgG Horseradish Peroxidase-linked whole antibody[o] at a dilution 1/3000. Proteins were visualized with a chemiluminiscent substrate[u] and after exposure to a film.[o]

Validation of the PENIA and PETIA

Serum and uCysC were analysed with PENIA[v] using the nephelometer[w] and PETIA[x] using the Cobas analyser.[y] PENIA and PETIA measure proteins in biological samples after reaction with a specific antiserum or reagent, resulting in complex formation in suspension. A light signal, emitted by an infrared diode (wavelength 840 nm), passes through the suspension, and a portion is scattered forward by the complexes, where it is focused onto a photodiode by an optical lens system. The particles are used if the antigen-antibody reaction does not permit sufficient formation of complexes to scatter the light. The voltage signal, which is directly proportional to scattered light intensity, is converted to digital units (“bits”) and compared to the corresponding values for the standard curve.

1. <u>Assay sensitivity</u>

The assay sensitivity was calculated based on the mean and corresponding standard deviation (SD) of the assay diluent (blank sample) analysis to determine the lowest concentration of sCysC and uCysC that can be measured.[29] According to an earlier report,[29] the limit of detection (LOD) was then calculated as two times the SD above the mean blank sample value which was obtained from 20 replicate measurements.

2. Imprecision

Serum and urine samples of six healthy cats and six CKD cats were analysed in duplicate on the same day and on three consecutive days. The intra-assay coefficient of variation (CV) was determined by dividing the SD of the parallel measurements on the same day by their mean and then multiplied by 100.[29] The inter-assay CV was determined likewise from the measurement on three consecutive days. [29]

3. Linearity

The method accuracy was assessed by evaluation of the linearity under dilution.[29] One serum and one urine sample with high CysC concentration were serially diluted four times. The observed serum and urinary CysC concentrations were plotted against the expected concentrations, and linear regression analysis was performed to calculate the corresponding correlation coefficient.

Measurement of sCysC and uCysC in healthy cats and cats with CKD

After validation of the nephelometric and turbidimetric assay for feline CysC, serum and urine sample volumes of 250 µL were analysed with the assay with the best validation parameters. During the analysis, room temperature was constant at approximately 22 °C. Urinary CysC was expressed as a ratio to the urinary creatinine (uCr) concentration, to compensate for differences in urine flow rates.[30]

Statistical analysis

Analyses were performed with a commercial software tool[z]. Descriptive statistics, Kolmogorov-Smirnov and Shapiro-Wilk tests were used to check the data for normal distribution. For parameters that approached a normal distribution[31] (age, USG, sCr, serum urea, sCysC and uCysC/uCr ratio), the Student's *t*-test was used to test for significant differences between the healthy cats and cats with CKD. For non-normally distributed parameters (body weight, UPC and uCr), the Mann-Whitney *U* test was used. The level of significance was assigned at $P < 0.05$.

Results

Study population

In total, 20 client-owned cats were recruited (age range: 1.8–19 years). Breeds included one Ragdoll, one Oriental Shorthair and 18 Domestic Shorthair cats. Eight cats were male neutered and 12 female neutered.

Ten cats were considered healthy, and 10 cats with CKD were included. Among the latter, two cats had IRIS stage 2 CKD, one of which had proteinuria (UPC > 0.40), three cats had IRIS stage 3 CKD, one cat had no proteinuria and one had borderline proteinuria (UPC 0.2–0.4) and one had severe non-renal proteinuria (UPC = 2.07), with a positive urine culture (*Escherichia coli*). Five cats had IRIS stage 4 CKD, with one cat borderline proteinuric, and the other four proteinuric (UPC > 0.40). One cat of the IRIS stage 3 group had hematuria (> 200 RBC/high-power field) and a positive urine culture (*Escherichia coli*).

Age, body weight, USG, UPC, serum urea, sCr, and uCr were compared between the healthy cats and cats with CKD (Table 1). Urine specific gravity was significantly lower in cats with CKD ($P < 0.001$), whereas UPC ($P = 0.002$), serum urea ($P = 0.008$), sCr ($P = 0.001$), and uCr ($P < 0.001$) were significantly higher in CKD cats compared with values in healthy cats. No statistical differences were observed for age and body weight.

Table 1. **Overview of the clinicopathological findings in 10 healthy and 10 CKD cats.** Variables that approach normal distribution are expressed as mean ± standard deviation (SD). Non-normally distributed parameters are expressed as median (interquartile range).

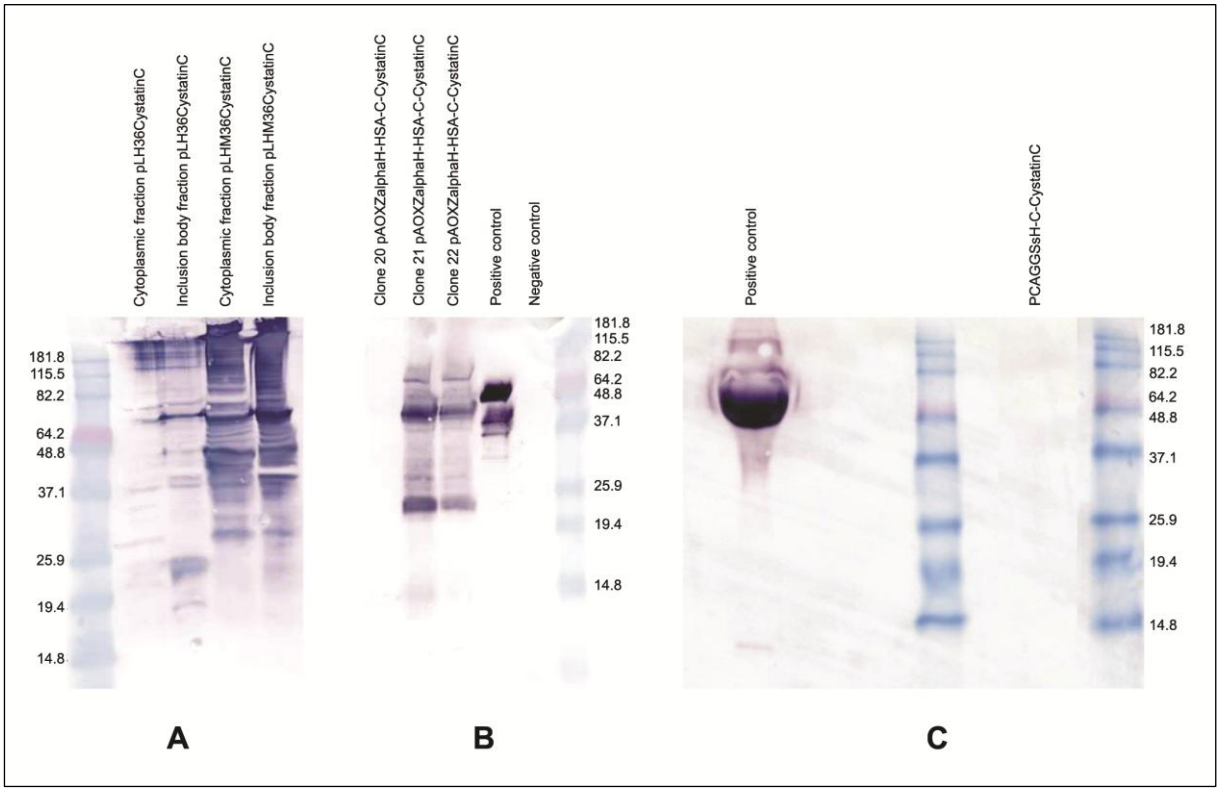
Variable	**Healthy cats (*n* =10)**	**Cats with CKD (*n* =10)**
Age (years)	6.95 ± 5.22	9.8 ± 4. 9
Body weight (kg)	4.50 (1.59)	3.6 (2.1)
USG	1.049 ± 0.011	1.013 ± 0.003
UPC	0.15 (0.06)	0.6 (1.1)
Serum urea (mmol/L)	9.39 ± 2.48	43.5 ± 29.5
sCr (µmol/L)	103.71 ± 12.92	540.8 ± 251.9
uCr (µmol/L)	21879.00 (12486.70)	3270.9 (1193.4)

CKD, chronic kidney disease; SD, standard deviation; USG, urine specific gravity; UPC, urine protein:creatinine ratio; sCr, serum creatinine; uCr, urinary creatinine.

Cloning of CST3 and protein purification

Three of the six clones possessed a sequence coding for CST3, 100% identical to what has been described earlier.[25] After expression in *Escherichia coli*, no protein band could be detected at the expected molecular weight of CysC, 13 kDa. Also electrophoresis results from *Pichia pastoris* and the HEK293T mammalian cells could not demonstrate CysC (Fig 2).

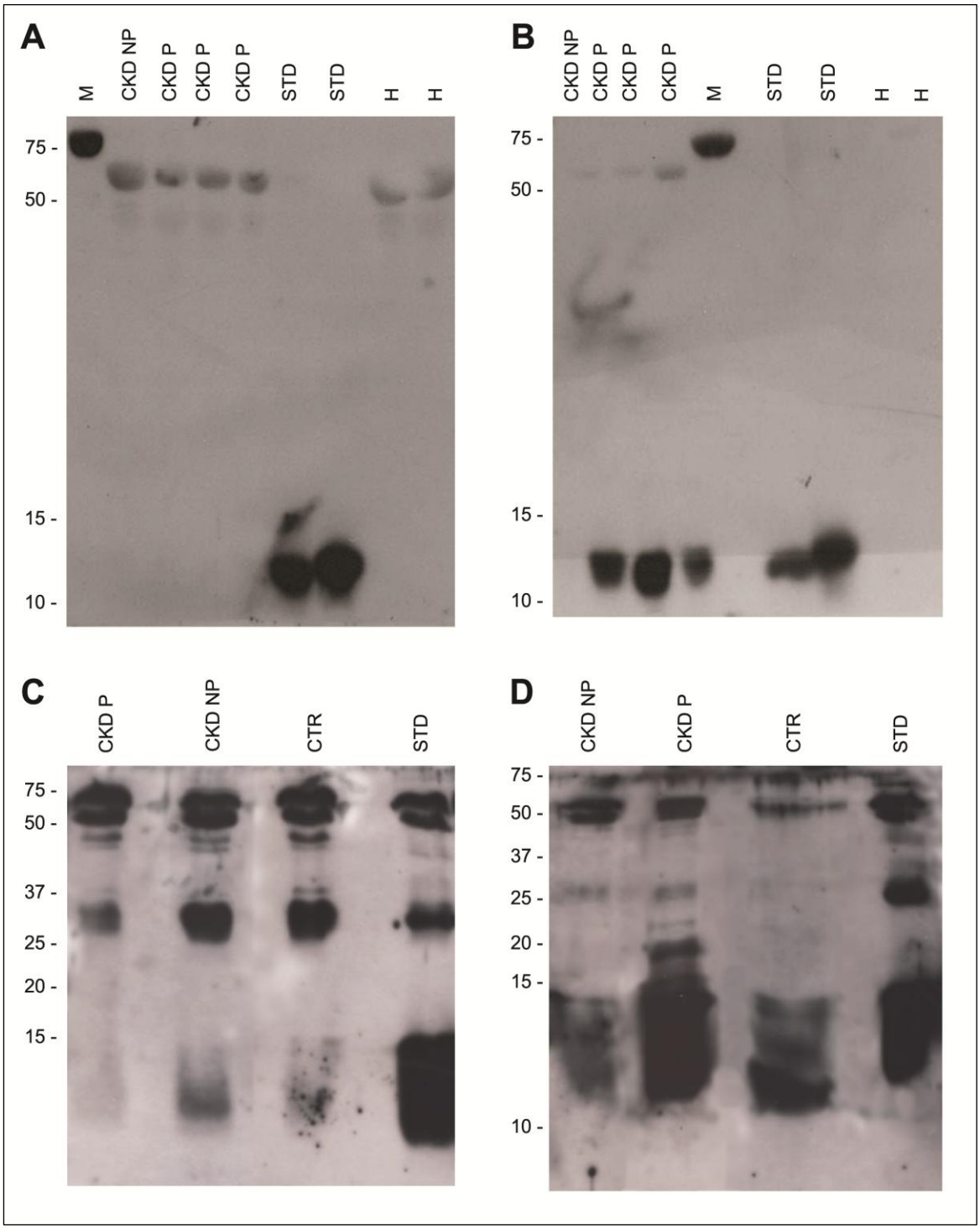
Figure 2. Gel electrophoresis results of the protein purification expression vector *Escherichia coli* (A), *Pichia pastoris* (B) and HEK293T mammalian cells (C). Recombinant CysC protein could not be demonstrated.



Western blot analysis

On Western blot analysis with antibodies from PENIA, a band was visible in urine at the expected CysC MM of approximately 13 kDa in the CKD cats with proteinuria, but not in the healthy cats nor in the cat with CKD without proteinuria (Figure 3B). A less prominent band was also visible at the molecular weight of approximately 52 kDa, which was not seen in the human CysC standard. In serum blots, however, there were no bands visible at the molecular weight of approximately 13 kDa, only light bands at approximately 52 kDa, both in the healthy and CKD cats (Figure 3A). On Western blot analysis with feline samples and antibodies from PETIA, on the other hand, a small band was visible in serum in one CKD cat at the expected MM of 13 kDa, but as for PENIA, small bands were also visible at approximately 26 and 52 kDa. This could also be observed at the standard (Fig 3C). For urine, a small band was more clearly visible at the expected MM of 13 kDa and was more pronounced in the proteinuric CKD cat. There was no difference in intensity of the bands between the healthy cat and the non proteinuric cat (Fig 3D).

Figure 3. Western Blot analysis with chemiluminiscent detection of the polyclonal rabbit anti-human cystatin C antibody from the PENIA in feline serum **(A)** and urine (**B**); Western blot analysis with chemiluminiscent detection of the polyclonal rabbit anti-human cystatin C antibody from the PETIA in feline serum **(C)** and urine **(D)**.



Numbers indicate the molecular mass (kDa); STD, human purified Cystatin C; M, colorimetric marker; H, healthy cat; CKD/NP, non-proteinuric cat with chronic kidney disease; CKD/P, cat with chronic kidney disease and proteinuria.

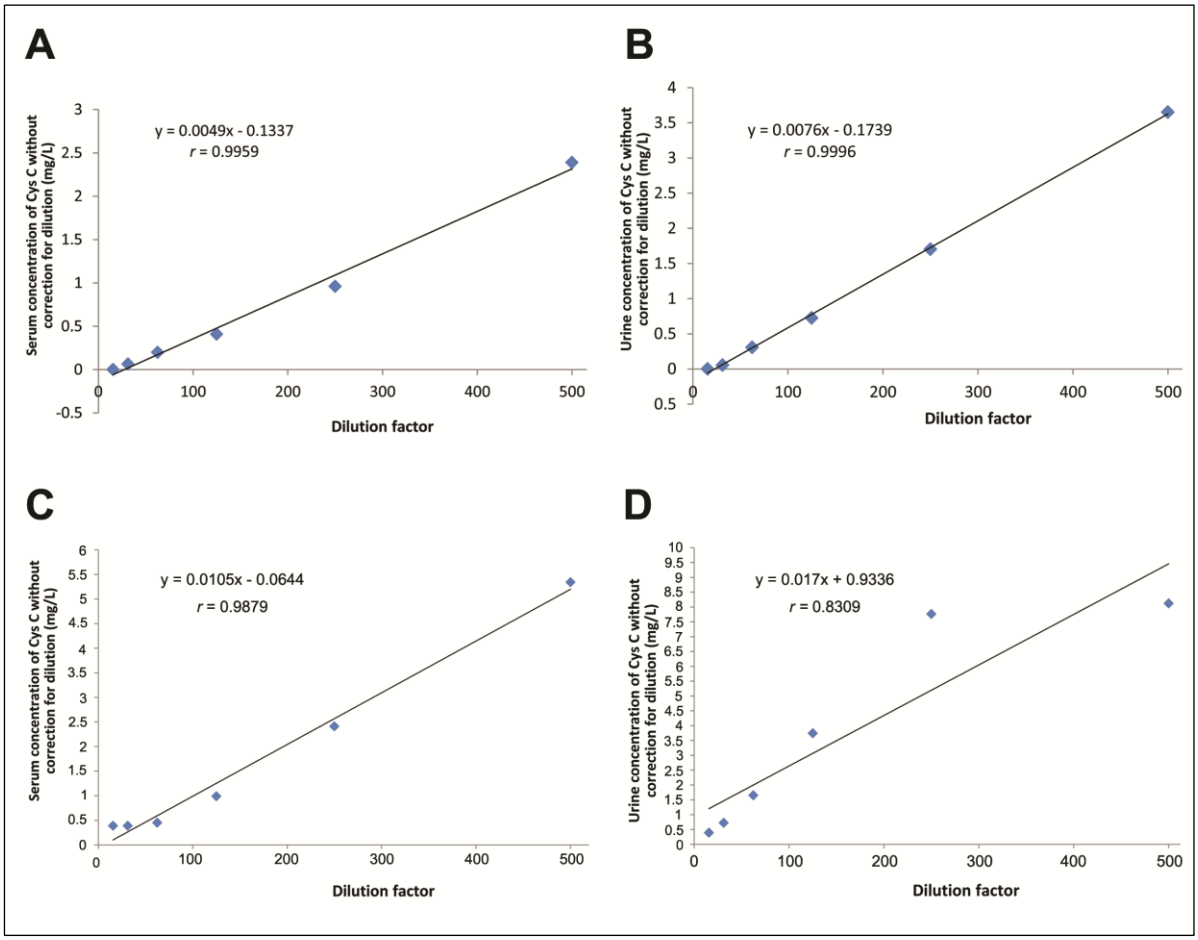
Validation of the PENIA and PETIA

The inter-and intra-assay CVs for CysC analyses in serum with PETIA and PENIA were comparable. For PETIA the inter-assay CV appeared even better. For urine on the other hand, the inter-assay CV was higher for PETIA than for PENIA. In addition, the LOD was high for PETIA. (Table 2). Regression analysis after serial dilution of a serum sample and urine sample showed a linear relationship between the observed and the expected uCysC and sCysC concentration for PENIA (Table 2; Figure 4A; Figure 4B), but the relation was less linear for PETIA (Table 2; Figure 4C, 4D).

Table 2. Validation parameters of the turbidimetric immunoassay compared with the nephelometric assay (both $n = 6$). The validation parameters for the PENIA appeared better than PETIA.

	Validation parameter	**PETIA**	**PENIA**
	Sensitivity Limit of detection (mg/L)	 0.39 mg/L	 0.049 mg/L
Serum	*Precision* Intra-assay coefficient of variation (%) Inter-assay coefficient of variation (%)	 0.8 9.5	 1.3 12.5
Urine	*Precision* Intra-assay coefficient of variation (%) Inter-assay coefficient of variation (%)	 0.4 15.9	 0.4 4.1

Figure 4. Sequential dilution of serum and urine of a cat with chronic kidney disease (CKD) analysed with PENIA **(A, B)** and PETIA **(C, D)** to evaluate linearity.



Cys C, Cystatin C; *r*, correlation coefficient.

Pilot study on sCysC and uCysC in healthy cats and cats with CKD

Based on the validation observations, but also due to practical reasons, PENIA was used for comparison of sCysC and uCysC/uCr ratio between 10 healthy cats and 10 cats with CKD (Table 3). The accuracy of the determined concentrations is unknown, as a purified feline CysC standard was not available at the time of measurement. Serum CysC (P = 0.02) and uCysC/uCr (P = 0.005) were significantly higher in CKD cats compared with the values in healthy cats. Urinary cysC was < LOD in all healthy cats, and in one CKD cat. The cat with a urinary tract infection (UTI) showed a high uCysC/uCr ratio (1282.3 mg/mol) compared to the mean uCysC/uCr ratio (612.63 mg/mol), but it was comparable to another CKD cat without UTI (1271.9 mg/mol).

Table 3. Relative sCysC and uCysC/uCr concentrations in 10 healthy and 10 CKD cats. As the parameters approach normal distribution, they are expressed as mean ± SD.

Parameter	**Healthy cats (*n* = 10)**	**Cats with CKD (*n* =10)**
sCysC (mg/L)	0.74 ± 0.20	1.42 ± 0.68
uCysC/uCr (mg/mol)	< LOD	645.1 ± 539.4

Discussion

This study had several important findings. We were not able to produce recombinant feline CysC with *Escherichia coli*, *Pichia pastoris* or mammalian cells as vector. We demonstrated that the human nephelometric and turbidimetric assay can be used to measure feline CysC, both in serum and urine. However, validation parameters of PENIA appeared to be better. Therefore, PENIA was used for analysis of sCysC and uCysC in both CKD and healthy cats. Both were significantly higher in CKD than in healthy cats.

Despite an earlier report,[25] we were not able to produce feline recombinant CysC with *Escherichia coli* as vector. Also with the other vectors, *Pichia pastoris* and the mammalian cells, feline recombinant CysC could not be produced. An early transcription or translation stop could explain our findings. Based on our experiments, a feline CysC standard could not be produced and therefore the human CysC standard needs to be used. This means that the obtained CysC concentrations are only relative values and we cannot evaluate which proportion of feline CysC is detected with the human assays.

On Western blotting, a protein band of approximately 13 kDa was observed in urine with PENIA antibodies, and both in serum and urine with PETIA antibodies. On all blots, a weaker band of approximately 26 and 52 kDa was also visible in serum and urine. According to an earlier report,[19] a similar molecular weight band was also seen in Western blots of pooled serum samples from people, dogs, and cats, developed with a polyclonal rabbit antihuman CysC antibody.[19] Nevertheless, there are some remarks. Firstly, on the PENIA blot, a major band at 13 kDa was observed in urine, but not in serum. On the PETIA blot, a small band could only be observed in serum from the non-proteinuric cat. This cat was in IRIS stage 3, and the proteinuric cat in IRIS stage 4. One would expect the signal to be stronger in the IRIS stage 4 CKD cat. For both PENIA and PETIA, signals were stronger in urine than in serum. A possible explanation is the higher CysC concentration in urine compared with that in serum. However, also in serum samples with high CysC concentrations, the 13 kDa band was absent. Alternatively, a difference in molecular structure between the urinary and serum form of feline CysC could explain the stronger signal in urine. Cystatin C inhibits cysteine peptidase through a substrate-competing mechanism.[32] Studies in rats and people have indeed shown that only the free and thus low molecular weight form of CysC

passes through the glomerulus, and that only the free form can be detected in urine.[9,11] A homology of 70% between feline and human CysC has been reported.[25,33] The epitope sequence to which the polyclonal rabbit anti-human CysC antibodies bind is not provided by the manufacturers of PENIA and PETIA, so a further evaluation of the difference of cross-reactivity between the antibodies against feline sCysC and uCysC is not possible. This could also explain the weak but positive signal on the serum blot with PETIA antibodies in contrast with PENIA.

PENIA showed a good precision and good linearity for feline sCysC and uCysC. For feline sCysC, the inter-assay CV of 12.5% was considered acceptable.[34] For PETIA on the other hand, the inter-assay CV for uCysC was higher than 15%, which is still acceptable, but far less ideal than PENIA.[34] Also LOD was high compared to PENIA, impeding CysC measurement in urine, in which low concentrations are expected. A less linear relationship than for PENIA between the observed and expected sCysC and uCysC could be observed after serial dilution of a serum and urine sample. Although two previous studies have evaluated feline CysC in plasma by a human turbidimetric assay,[35] and in serum by a human nephelometric assay,[36] no validation data were documented. Validation data provided by the manufacturer of the nephelometric assay for human serum are an intra-assay CV range of 1.5–3.1%, and an inter-assay CV range of 1.5–3.5%, and for the PETIA an intra-assay CV of 0.6%–2.7% and an inter-assay CV of 1.9%–3.8%. Several studies have reported an intra-assay CV for the PENIA ranging from 2.0 to 7.1% and an interassay CV ranging from 2.6 to 7.9% for human urine.[12,13,37] For the PETIA, validation reports are available for dogs, with intra-assay CVs ranging from 0.9% to 2.4% and inter-assay CVs ranging from 1.7% to 9.6% for serum. [18,19,38] For urine, only one validation report for PETIA is available with mean intra-assay and inter-assay CV of 6.4% and 2.9% respectively.[14] Matrix interferences cannot be excluded, but are not provided by manufacturers of both PENIA and PETIA. Based on our findings and a similar study in dogs,[39] we recommend using PENIA for CysC evaluation in cats.

The PETIA had a high LOD and was somewhat less accurate. Based on these observations, but also due to practical reasons, we analysed serum and urine samples of 10 healthy and 10 CKD cats with PENIA, to compare sCysC and uCysC. We detected a highly significant difference in sCysC concentration between healthy cats and cats with CKD. However, some overlap in the concentrations of both groups was noticed. As we did not

perform a GFR measurement in these cats, we cannot exclude that some of the healthy cats had early renal disease. The one CKD cat with UTI in our study also did not have a higher uCysC/uCr in comparison with other CKD cats. In people[37] and dogs,[12] hemoglobin did not interfere with the measurement of uCysC. However, to the authors' knowledge, there are no reports about the measurement of uCysC in patients with lower UTI.

Our results are in accordance with an earlier study.[36] In contrast, plasma CysC in CKD cats did not exceed a relatively broad reference interval determined with a turbidimetric assay reported earlier for a group of healthy cats.[35] As GFR was not determined,[35] early, but undiagnosed, kidney impairment cannot be ruled out in apparently healthy animals. Other explanations for the differences include the different types of assays used for CysC determination.

Based on our results, the results of an earlier study, and studies in dogs and people, we suggest that sCysC may be a promising marker for GFR estimation in cats. As the nephelometric assay is commercially available and requires only 250 µL of serum, it can be automated, which is a major advantage. Further studies are needed to evaluate if sCysC has a similar superiority over sCr in detecting early kidney damage in cats, as reported in people and dogs.

Urinary CysC levels were below the limit of detection in the healthy cat group, and CKD cats had significantly higher uCysC/uCr than the healthy cats. Cystatin C is a small protein that is completely reabsorbed and catabolized in the proximal tubules. Detectable urinary concentrations thus reflect tubulointerstitial damage.[40] Other markers for tubular damage, such as N-acetyl-b-D-glucosaminidase (NAG) and retinol-binding protein (RBP), have previously been measured in feline urine.[41,42] For RBP, our research group validated and applied a human ELISA kit in cats.[42,43] There are important advantages of uCysC as renal marker over uRBP and uNAG. Firstly, the nephelometer technology (uCysC) is less time-consuming than an ELISA (uRBP) or a nonautomated colorimetric technique (uNAG). Secondly, uNAG seemed to overlap between healthy cats and cats with CKD, and the assay showed a very high inter-assay CV.[41]

A limitation of this study was the small number of patients included. Additional studies with more patients, GFR measurement, and the assessment of diagnostic sensitivity

and specificity are required for further evaluation of CysC as a marker in feline renal medicine.

Conclusion

In conclusion, this study is the first report on the validation of a nephelometric and turbidimetric assay to measure CysC in serum and urine of healthy cats and cats with CKD. Based on the validation results, we recommend to use PENIA for uCysC measurement in cats. Serum CysC can be analysed both with PENIA and PETIA. A significant difference in sCysC and uCysC between CKD and healthy cats could be observed. However, additional studies are required.

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End Notes

[a] Advia 2120, Siemens, Brussels, Belgium

[b] Architect C16000, Abbott Max-Planck-Ring, Wiesbaden, Germany

[c] Iricell velocity, chemical system and IQ 200 SPRINT, microscopic system; both from Instrumentation Laboratory, Zaventem, Belgium

[d] Immulite 2000 system, Siemens Healthcare Diagnostics, Marburg, Germany

[e] Wask Copan, MLS, Vitek 2 system, BioMerieux, Brussels, Belgium

[f] Total RNA Isolation Reagens, Abgene, Surrey, United Kingdom

[g] Nanodrop 1000, Isogen, De meern, the Netherlands

[h] Improm II Reverse Transcriptase kit, Promega, Madison, WI, USA

[i] Fastart Taq DNA, Roche Diagnostics, Penzberg, Germany

[j] Geneclean II-kit, Q-biogene, Irvine, USA

[k] Invitrogen, California, USA

[l] BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Thermofisher Scientific, Waltham, MA, USA

[m] 3130xl DNA Analyser, Applied Biosystems, Thermofisher Scientific, Waltham, MA, USA

[n] BioEdit, Ibis Biosciences, Abbott, Carlsbad, CA, USA

[o]Amersham Biosciences, GE Healthcare Europe GmbH, Diegem, Belgium

[p] Biorad, Nazareth-Eke, Belgium

[q] Siemens Healthcare Diagnostics, Marburg, Germany

[r] Dako, Glostrup, Denmark

[s] all from Sigma-Aldrich, St.Louis, MO, USA

[t] personal gift from Dr. Harald Althaus, Siemens Healthcare Diagnostics

[u]Perkin Elmer, Zaventem, Belgium

[v] particle-enhanced nephelometric immunoassay, Siemens Healthcare diagnostics, Marburg, Germany

[w] Behring Nephelometer (BN) ProSpec, Siemens Healthcare Diagnostics, Marburg, Germany

[x] particle-enhanced turbidimetric assay, Dako Glostrup, Denmark

[y] Cobas C system, Roche Diagnostics Gmbh, Mannheim, Germany

[z]SPSS 20, IBM SPSS software, IBM Corporation, Armonk New York, USA

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CHAPTER IV

BIOLOGICAL VALIDATION OF FELINE CYSTATIN C

SECTION §4.1

THE EFFECT OF FEEDING, STORAGE AND ANTICOAGULANT ON FELINE SERUM CYSTATIN C

Liesbeth F.E. Ghys[1], Dominique Paepe[1], Hervé P. Lefebvre[2], Elien R.L. Taffin[1], Myriam Hesta[3], Joris R. Delanghe[4], Pascale Smets[1], Veerle Vandendriessche[3], Sylvie Daminet[1]

[1] Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium

[2] Unité de Recherche Clinique, École Nationale Vétérinaire de Toulouse, Université de Toulouse, Chemin des Capelles 23, Toulouse Cedex 3, France

[3] Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, B-9820, Merelbeke, Belgium

[4] Department of Clinical Chemistry, Microbiology and Immunology, Faculty of Health Medicine and Life Sciences, Ghent University, De Pintelaan 184, B-9000, Ghent, Belgium

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Summary

Serum cystatin C (sCysC) is a possible marker for early detection of chronic kidney disease (CKD) in cats. Further biological and clinical validation are required. The objective of this study was fourfold. The first two aims were to investigate if food intake and/or circadian rhythm affect feline sCysC. The third aim was to study the stability of sCysC at different storage conditions and the effect of an extra freeze-thaw cycle. The fourth aim was to investigate if plasma CysC (pCysC) differed from sCysC. A crossover study with 10 healthy laboratory cats fed the same commercial dry food was performed to study the influence of feeding and diurnal variation. Four CKD and three healthy cats were included for the storage study and 17 CKD and 10 healthy cats for comparison of pCysC with sCysC.

A significant daily sCysC variation was seen. Pre- and postprandial sCysC and sCr concentrations did not change significantly. Serum CysC significantly increased during storage at room temperature. After freezing, sCysC significantly decreased after five and 12 months at both -20 °C and -72 °C. Plasma CysC was significantly lower than sCysC.

These findings suggest that it is not mandatory to fast cats before evaluation of sCysC and sCr. Samples were stable during routinely used storage conditions. Freezing is not recommended for longer than five months, based on our findings. However, additional studies are required to value the clinical relevance of decreased sCysC observed after prolonged storage. Plasma and serum CysC cannot be compared directly.

Keywords: Cat; Chronic kidney disease; Meal; Plasma, Serum; Temperature

Introduction

Chronic kidney disease (CKD) is one of the most common feline diseases of geriatric cats. Early diagnosis and subsequent treatment of CKD aids in improving quality of life and longevity.[1,2] Since clearance tests to evaluate glomerular filtration rate (GFR) are impractical to perform,[3] the indirect GFR markers, serum creatinine (sCr) and urea, are measured routinely. These both lack sensitivity and specificity for early diagnosis of feline CKD.[3] Therefore, indirect markers that can reliably and easily evaluate kidney function in cats are required.

Cystatin C (CysC) is a low molecular mass (MM) protein (13 kDa). Studies in rats have demonstrated that CysC fulfils the required properties for an endogenous GFR marker.[4] Our research group has validated the human particle enhanced nephelometric immunoassay (PENIA) (BN Prospec, Siemens) for determination of feline CysC in serum and urine.[5] In a pilot study serum CysC (sCysC) was significantly higher in cats with CKD compared to healthy cats. These findings make it an interesting renal marker. However, further investigation is warranted to evaluate the diagnostic value of sCysC as a renal biomarker in cats.

One of the shortcomings of sCr is the effect of food intake. Dietary meat intake increased sCr both in dogs[6] and cats.[7] However, sCysC is not influenced by food intake in humans,[8,9] in contrast with dogs.[10] In addition, a circadian variation of sCr has been observed for both humans[11] and dogs,[12,13] although the magnitude of these variations was minor and probably not clinically relevant.[3,11] For sCysC, a circadian variation has been observed in dogs,[14] but not in humans.[11] To the authors' knowledge, studies in cats evaluating the effect of feeding and circadian rhythm on sCysC and comparison with sCr are not yet available.

For use in practice, it is important to know the appropriate storage conditions for samples before measurement of CysC. To the authors' knowledge, the stability of feline CysC during storage has not yet been studied. In humans[15] and dogs,[14] sCysC seemed to remain stable after storage for six and 12 months respectively at -80 °C.

Cystatin C is mostly measured in serum. Some studies in dogs[10] and cats[16] have measured CysC in plasma instead of serum. To the authors' knowledge, no studies compared sCysC with plasma CysC (pCysC). However, this information would be useful in clinical practice.

The purpose of this study was fourfold. The first two aims were to investigate the effect of feeding and diurnal variation on feline sCysC compared with sCr. Thirdly, the stability of feline sCysC was investigated during storage at room temperature (RT) for one day, at 4 °C for one day and one week, and at -20 °C and -72 °C for five months and one year. The effect of an extra freeze-thaw cycle on samples stored at -20 °C and -72 °C was also investigated. Finally, concentrations of CysC measured in plasma and serum were compared.

Materials and methods

Study population

To determine the effect of feeding and daily variation on sCysC and sCr, healthy laboratory domestic short- or longhair (DSH/DLH) cats were included. For the storage study and comparison of sCysC and pCysC, client-owned CKD and healthy cats were included. Inclusion criteria for the healthy cats were: no disease history and no clinically relevant abnormalities detected upon physical examination, complete blood cell count (CBC), serum biochemistry profile (including total thyroxine concentration) and urinalysis. Cats with body condition score (BCS) < 4 or ≥ 6 on a 9-point scale[17] were excluded for the food study. For the other study under- and overweight cats (BCS 3–7) could be included. Cats with urine specific gravity (USG) < 1.035 could only be included if concurrent azotemia was absent, i.e. sCr < 161.8 µmol/L (see Chapter IV §4.2). Cats were excluded if they received medication that could influence kidney function such as non-steroidal anti-inflammatory drugs (NSAIDs), angiotensin converting enzyme (ACE)-inhibitors or angiotensin receptor blockers (ARB), from the month before the study-onset onwards.

The diagnosis of CKD was based on the presence of compatible clinical signs, persistent renal azotemia and USG < 1.035. Only CKD cats with International Renal Interest Society (IRIS) stage 2 or higher were considered for this study. Exclusion criteria for cats with CKD were evidence of concurrent systemic disease based on the history, physical examination, CBC, serum biochemistry profile and urinalysis. Treatment with ACE-inhibitors or ARB was stopped one week before the onset of the study. Treatment with a renal diet could be continued. Both CKD and healthy cats were excluded if they had received NSAID's, corticosteroids, antibiotics or β-blocking agents within one month prior to inclusion.

Study design

The effect of feeding versus fasting and diurnal variation was tested in a crossover study that lasted 43 days. Samples were taken on day 34 and day 43. All cats received a standard maintenance diet (Hill's Science Plan feline Adult Optimal Care Chicken Dry) for the duration of the study. All cats were fed an amount of energy required to maintain ideal body weight. The estimated amount of food was calculated as the sum of the metabolic weight and the energy factor for active cats as indicated in the guidelines of the European Pet Food Industry Federation (Fediaf) and adapted to maintain a steady body weight. Cats were randomly divided in two groups: group A and B. Pre-prandial samples were taken from all cats after fasting overnight for at least 16 h. During the experimental procedure, water was offered ad libitum to all cats. Group A was fed after the first blood sample was drawn, while group B remained fasted for 10 h. One week later, group B cats were fed after the first blood sample was drawn and group A was fasted. Cats that were fed, had access to food for one hour. The amount of food eaten was recorded. The other blood samples were taken 30 min, 1 h, 2 h, 3 h, 4 h, 6 h, 10 h and 12 h after the meal was taken away in both group A and B, according to a similar study in dogs.[10]

For the storage study and comparison of sCysC and pCysC, both CKD and healthy cats were fasted the night before the sampling, but water was accessible. The protocol of the studies was approved by the Local Ethical and Deontological Committee of the Faculty of Veterinary Medicine, Ghent University (EC2011_197; Date of approval January 30, 2012). All owners whose animal participated in the study gave written informed consent.

Procedures

A standard physical examination, including thyroid gland palpation as previously described,[18] was performed in all cats prior to inclusion. For the laboratory cats, this was repeated on day 33 of the crossover study. Additionally, systolic blood pressure (SBP) was measured using the Doppler ultrasonic technique according to the ACVIM consensus guidelines.[19] Cats were considered hypertensive if SBP exceeded 160 mmHg.[19,20] To perform complete blood examination, two mL of blood were taken by jugular venipuncture using a 23 G needle. After centrifugation (5 min at 1931x *g*), serum was analyzed the same day. The CBC[a] and serum biochemistry[b] were performed in all cats. Urine (5 mL) was collected by ultrasound guided cystocentesis with a 22 G needle. Urine specific gravity was determined

using a manual refractometer. Further urinalysis consisted of a urinary dipstick test[c], measurement of the urinary protein: creatinine ratio (UPC)[d], sediment analysis and bacterial culture[e]. Urine was centrifuged (3 min at 355 x *g*) and the urinary sediment was analyzed within 30 min as previously described.[21]

During the feeding study, additional blood samples were taken with a 25 G needle. On the day the samples were taken, the amount of food that was eaten was recorded. Blood was centrifuged (5 min at 1931x *g*) and serum was divided in aliquots of 300 µL and stored at -72 °C until batched analysis. Samples of the feeding study were analysed within four days after sampling. Group A and B were compared in the crossover design.

For the storage study, 4 mL of blood were taken additionally and centrifuged for 5 min at 1931x *g*. Serum was divided in eight aliquots of 300 µL and stored at room temperature (RT) (20 °C), at 4 °C, at -20 °C and at -72 °C. Within 3 h, CysC was measured in one aliquot of serum stored at RT (T0 sample). Twenty-four hours after sampling, one aliquot of serum stored at 20 °C (T_{24h}RT) and 4 °C (T_{24h}4 °C) was analysed. Furthermore, sCysC was determined after one week of storage at 4 °C (T_{1w}4 °C), after five and 12 months at -20 °C (T_{5m}-20 °C and T_{12m}-20 °C) and after five and 12 months at -72 °C (T_{5m}-72 °C and T_{12m}-72 °C). After measurement of sCysC in T_{12m}-20 °C and T_{12m}-72 °C, the aliquots were refrozen at -20 °C and reanalysed the next day, to assess the effect of an extra freeze-thaw cycle. At the several time points, serum samples were analysed in batch, and the same reagent with the same lot number was used.

To compare sCysC with pCysC, 2 mL of blood were taken additionally in an EDTA- and serum tube that were centrifuged (5 min at 1931 x *g*). Plasma and serum aliquots of 300 µL were stored at -72 °C until batched analysis.

Serum feline CysC was determined using PENIA[f], previously validated for feline CysC by our group.[5] Serum Cr was analyzed in the food study with an enzymatic assay[g], previously validated for cats by our group.[22] Serum Cr was analyzed in the other studies with a modified Jaffe assay, validated by a veterinary commercial laboratory, with a RI of (64.5–161.8 µmol/L) (see Chapter IV §4.2).

Statistical methods

Statistical analyses were performed using statistical software[h]. The level of significance was set at 0.05. All data were analysed with a general linear model. If a significant time effect was observed, a Tukey's test was used as post-hoc test. Interactions between the status (food versus fasted) and time points were tested for the feeding study. If the interactions were not significant, the time to time comparison was performed globally. If the interactions were significant, the time effect was analysed separately (fasted or meal). Bland-Altman plots were designed to compare sCysC with pCysC.

Results

Study population and procedures

Ten healthy laboratory DSH/DLH cats were included to study diurnal variation and the effect of feeding on sCysC and sCr. Mean ± standard deviation (SD) age of the complete group was 9.9 ± 2.2 years. Group A and B both consisted of five cats: four female neutered and one male neutered cat. The two groups in the feeding study did not significantly differ in age, bodyweight, SBP, heart rate, sCr, urea, USG and UPC. The breed and the number of females and males were identical in each group. The descriptive statistics of group A and B are presented in Table 1. Between day 1 and day 33, the day before blood sampling, 8/10 cats had lost weight i.e. median (range) 0.1 (0–0.6) kg. The bodyweight of the other two cats remained stable. During the blood sampling, only 4/10 ate the total amount of food required to maintain ideal body weight i.e. median (range) 75 (49–100)%.

To investigate the effect of storage temperature and duration, four cats with CKD and three healthy cats were included. One CKD cat was a British shorthair cat, all other cats were DSH/DLH cats. The CKD group consisted of four male neutered cats and the healthy cats of one male neutered and two female neutered cats. In the CKD group, three cats were in IRIS stage 2 and one cat was in IRIS stage 4. One cat was treated with ACE-inhibitors, but the therapy was stopped one week before sampling.

Plasma CysC and sCysC were measured in 10 cats with CKD and in 17 healthy cats. Six CKD cats were DSH/DLH. The others were a Burmese cat, a Ragdoll, a Siamese and a British shorthair cat. All healthy cats were DSH/DLH. The CKD group consisted of one female, two female neutered and seven male neutered cats. Seven of the 10 CKD cats were in IRIS stage 2, two in IRIS stage 3 and one in IRIS stage 4. In one of the CKD cats, ACE-inhibitor therapy was stopped one week before inclusion. The other CKD cats did not receive renin-angiotensin-aldosterone system (RAAS) modulating therapy.

Table 1. Descriptive statistics (mean ± SD) of healthy laboratory cats of group A (meal-fasted) and group B (fasted-meal) at T0. No statistical differences were observed. *P*-values have been added in brackets.

Variable	Group A (*n* = 5)	Group B (*n* = 5)
Age (years) (*P* = 1.0)	9.9 ± 2.3	10.0 ± 2.4
Bodyweight (kg) (*P* = 0.4)	3.8 ± 0.6	4.3 ±1.1
Heart rate (bpm) (*P* = 0.9)	166 ± 32	168 ± 19
SBP (mmHg) (*P* = 0.7)	154 ± 23	148 ± 24
sCr (µmol/L) (RI 64.5–161.8) (*P* = 0.6)	114.6 ± 11.4	120.0 ± 17.2
sCysC (mg/L) (*P* = 0.4)	0.83 ± 0.19	0.78 ± 0.12
Serum urea (mmol/L) (RI 6.2–12.7) (*P* = 0.08)	6.7 ± 0.9	7.9 ± 1.0
USG (*P* = 0.8)	1.037 ± 0.018	1.034 ± 0.015
UPC (*P* = 0.2)	0.18 ± 0.04	0.35 ± 0.23

SD, standard deviation; SBP, systolic blood pressure; bpm, beats per minute; sCr, serum creatinine; RI, reference interval; sCysC, serum cystatin C; USG, urine specific gravity; UPC, urinary protein:creatinine ratio.

Effect of feeding

There was no significant difference in mean sCysC and sCr concentration between fasted cats and cats that had been fed. Figure 1 and 2 present mean ± 1.96 x standard error of the mean of sCysC and sCr of the fed and unfed cats. There was no interaction between time and status (meal or fasted) for sCysC, therefore the time to time comparison of sCysC could be done globally. Serum CysC was significantly different between T0, 30 min ($P = 0.009$) and 4h ($P < 0.001$); between 30 min and 2 h ($P < 0.001$), 3 h ($P = 0.002$), 4 h ($P < 0.001$), 6 h ($P < 0.001$), 9 h ($P < 0.001$) and 12 h ($P < 0.001$); between 1 h and 2 h ($P = 0.01$), 4 h ($P <$

0.001), 6 h (P = 0.03), 9 h (P = 0.01) and 12 h (P < 0.001); and between 3 h and 4 h (P = 0.001). For sCr, there was a significant (P < 0.001) interaction between time and status. Therefore, time effect was analysed separately for each status. No time effect was observed in either status.

Figure 1. Mean serum CysC (sCysC) concentration with 95% CI (mean ± 1.96 x SEM) in 10 heathy cats fasted and after a meal. Although the figure suggests a significant difference, no effect of feeding on sCysC could be observed. The maximum and minimum sCysC concentrations were observed 36 and 246 min after the first sample respectively.

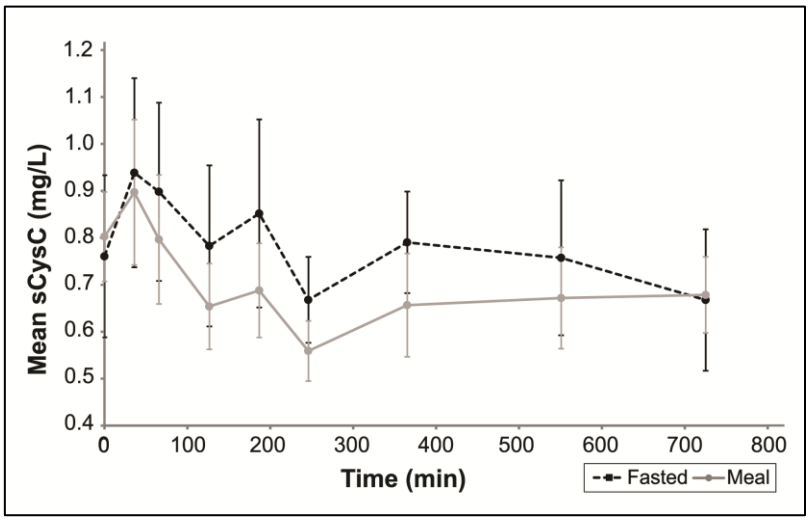
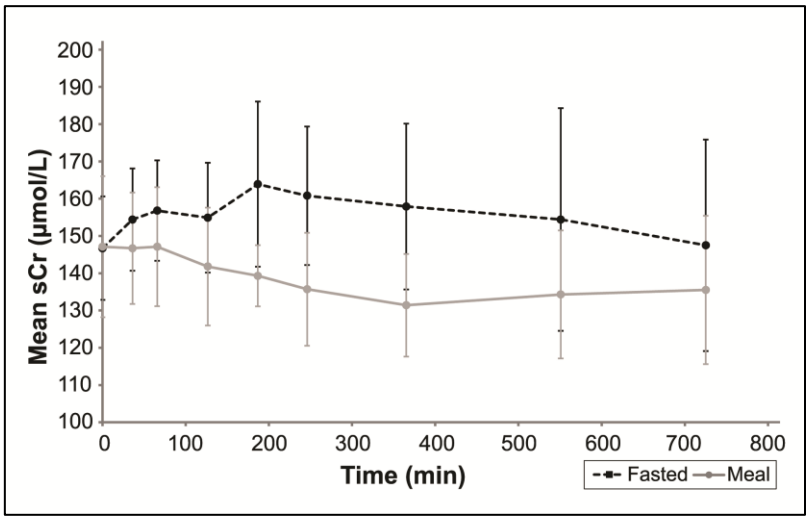


Figure 2. Mean serum creatinine (sCr) concentration with 95% CI (mean ± 1.96 x SEM) in 10 healthy cats fasted and after a meal. As for sCysC, no significant difference between fed and unfed cats can be observed.



Effect of storage

To determine optimal storage conditions, serum samples of all seven cats were analysed. The mean ± SD concentrations, the mean ± SD difference with T0 samples and the mean percentage increase and decrease are presented in Table 2 and Table 3. Serum CysC significantly differed from baseline values at room temperature (P = 0.03). After freezing, sCysC decreased and significantly differed from baseline values after five (P = 0.001) and 12 months (P < 0.001) at -20 °C, and after five (P = 0.002) and 12 months (P < 0.001) at -72 °C. The sCysC concentration was significantly higher in samples stored at -20 °C (P = 0.003) and -72 °C (P = 0.04) after an extra freeze-thaw cycle.

Table 2. Mean ± SD concentration and mean % increase or decrease of sCysC at different storage conditions. * indicates if the concentration is significantly different from T0 sample.

	T0	**T_{24h} RT**	**T_{24h} 4°C**	**T_{1w} 4 °C**	**T_{5m} -20°C**	**T_{5m} -72 °C**	**T_{12m} -20 °C**	**T_{12m} -72 °C**
sCysC (mg/L)	1.2 ± 0.5	1.3 ± 0.5*	1.3 ± 0.5	1.3 ± 0.5	1.1 ± 0.5*	1.1 ± 0.5*	1.0 ± 0.5*	1.0 ± 0.4*
Mean difference (mg/L)		0.02 ± 0.02	0.02 ± 0.02	0.03 ± 0.03	0.1 ± 0.07	0.1 ± 0.07	0.3 ± 0.07	0.3 ± 0.08
Mean % increase/decrease		1.9%	1.7%	0.6%	-10%	-10%	-21%	-21%

SD, standard deviation; sCysC, serum cystatin C; RT, room temperature.

Table 3. Mean ± SD concentration and mean % increase or decrease of sCysC and uCysC before and after freezing-thawing. * indicates if the sCysC and uCysC concentrations post freeze/thaw cycle significantly differ from before freeze/thaw cycle.

		Before freeze/thaw cycle	After freeze/thaw cycle	Mean ± SD difference	Mean % increase/ decrease
sCysC (mg/L)	**-20 °C**	1.0 ± 0.5	1.1 ± 0.5*	0.08 ± 0.04	8.3%
	-72 °C	1.0 ± 0.4	1.0 ± 0.5*	0.07 ± 0.07	6.7%

SD, standard deviation; sCysC, serum cystatin C.

Effect of anticoagulant

The medium effect (plasma or serum) was independent of the status effect (healthy or CKD). A highly significant effect of the medium on CysC was observed. Plasma CysC was significantly lower ($P < 0.001$) than sCysC in both healthy cats and cats with CKD. The Bland-Altman Plot shows wide limits of agreement (Figure 3), indicating that sCysC and pCysC were not equivalent. A trend could not be observed.

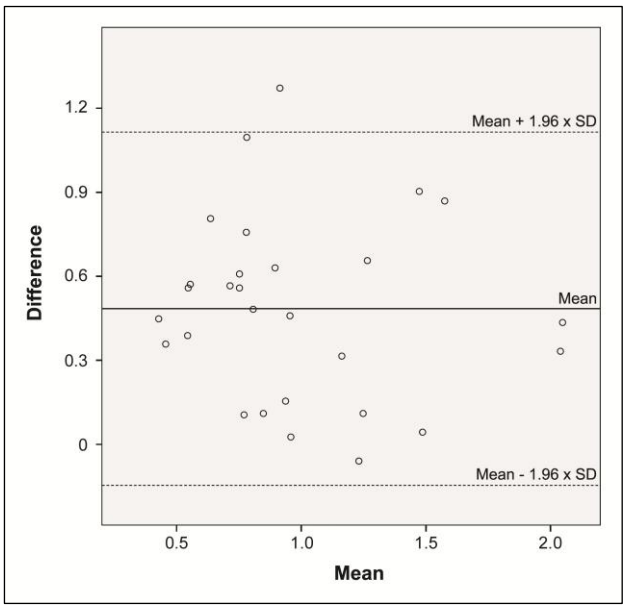


Figure 3. Bland-Altman Plot of plasma cystatin C (pCysC) and serum cystatin C (sCysC). SD, standard deviation. "Mean" is the mean CysC concentration in serum and plasma of the different samples respectively. "Difference" is the difference in CysC concentration between serum and plasma.

Discussion

Firstly, we demonstrated that feeding with commercial dry food does not influence sCysC and sCr in cats. We also observed that there was a significant daily variation of sCysC in contrast with sCr. Secondly, our study showed that feline sCysC decreases over time after freezing. Thirdly, we observed that pCysC is significantly lower than sCysC in cats.

To the authors' knowledge, this is the first study evaluating the effect of fasting versus feeding on sCysC in cats. Similar to human medicine[8,9] no effect of feeding on sCysC could be demonstrated. However, food can cause chylomicronaemia.[23] In humans, it has been shown that chylomicrons can cause interference with the PENIA,[24] but whether the same occurs in cats is not known. In dogs, feeding a commercial diet caused a dramatic decrease in pCysC concentration, which started the first hour after the meal and lasted for 9 h. After 12 h, pCysC returned to baseline values[10] Other authors described no effect of feeding on canine sCysC.[25] Braun and colleagues linked the decrease in pCysC to an increase in GFR, but the dietary effect on GFR has only been demonstrated in healthy dogs after consumption of raw[26] or cooked meat,[27] but not after a commercial diet. Studies evaluating GFR in healthy cats after consumption of a commercial diet or raw or cooked meat are not available.

Feeding did not affect sCr in the present study, which suggests that fasting prior to measurement of feline sCysC and sCr is not absolutely mandatory. In contrast to the present study, a postprandial increase[7] and decrease[28] in feline sCr have been described. Sagawa et al. postulated an increase in sCr in relation to the creatine content of the diet. Both in the present study and the study of Reynolds and colleagues, cats were given a commercial dry cat food with poultry meat meal as meat source. For dogs, commercial dry dog foods may cause an increase,[29] decrease[27] or no change in sCr.[30] The protein content of the diets of the present study and the study of Reynolds and colleagues corresponds with the normal protein requirement.[31] Reynolds and colleagues attributed a lower postprandial sCr concentration to increased GFR. However, unaltered sCr concentrations with increased mean GFR have been observed in cats, but these cats were fed a high protein diet.[32] To the authors' knowledge, no results are available in cats fed a normal protein diet. Therefore, it appears unlikely that feeding a diet with normal protein content causes an increased GFR leading to decreased sCr. Other explanations for different findings in the studies might be inter-individual differences

between cats and the amount of food provided. We observed large inter-individual differences in postprandial change in sCr concentration, which was also seen in dogs.[29]

We observed a significant time to time variation for sCysC, but not for sCr. Serum CysC significantly differed between the first samples, T30 and T60 respectively, and the evening samples. However, the changes in sCysC were only small. In addition, we also observed large discrepancies between individuals. Therefore, the diurnal variation in sCysC is probably not clinically relevant. Limited information is available about circadian variability of sCysC and sCr. In humans, higher sCysC concentrations were observed in the afternoon.[11] Reynolds and colleagues observed 18% higher sCr concentrations in the afternoon than in the morning in fed cats. In dogs on the other hand, lower sCr concentrations in the evening are seen in both fasted dogs and dogs receiving a meal once daily.[33] Additional studies with more cats and sampling over a longer period are required to investigate diurnal variations in blood parameters in cats.

This study showed that feline sCysC was significantly different after storage at RT for 24 h and after longer storage at -20 °C and -72 °C, compared to T0. However, the difference after storage at RT was small and probably not clinically relevant. In addition, very mild increases in sCysC were observed during the routinely used storage conditions, i.e. room temperature and 4 °C. Nevertheless, storage after five and 12 months caused a significant decrease in sCysC of up to 21%. To the authors' knowledge, no other studies have evaluated storage of feline sCysC. In dogs, sCysC appeared to be stable even after one year storage at -80 °C.[14] However, in the latter study, a serum pool was used, which makes comparison with the present study difficult. Freezing and thawing of the samples also caused a significant increase of the feline sCysC concentration. These findings are in contrast with human studies.[34]

We calculated the mean percentage increase and decrease of the samples at the different storage conditions (Table 2 and 3). The feline sCysC range of analytical variation is 17.4% (mean interassay coefficient of variation + 2x SD).[5,35] The decrease in sCysC after five months storage at -20 °C and -72 °C and the difference after freezing and thawing may be caused by between-day variation of the assay. During the feeding study, samples were stored for four days at -72°C before sCysC analysis. Since sCysC appeared to be stable for five months at -72 °C, we do not think that storage of the samples at -72 °C for four days could

have influenced the results. Longer storage at both -20 °C and -72 °C caused a further decrease of the sCysC concentrations, which cannot be solely attributed to assay variation. A limitation of our study is the small sample group. It is difficult to value the clinical relevance of our the observed decrease in sCysC following longer storage, since only two studies have evaluated sCysC in cats.[5,36] We also do not know if the variation at higher concentrations is different from low concentrations. Therefore, additional studies are warranted. However, until more data are available, storage of samples to evaluate sCysC is not recommended for longer than five months.

Plasma CysC was significantly lower than sCysC, both in cats with CKD and healthy cats. This indicates that the anticoagulant might influence sCysC concentration. However, we did not investigate the effect of freezing on pCysC compared to sCysC, which could have influenced our results.

This study is limited by the small sample size to investigate the different storage conditions of sCysC. Therefore, additional studies with more samples and different storage conditions are required. In addition, no GFR measurement was performed, thus early CKD in the healthy cats cannot be excluded.

Conclusion

For the first time, the effect of feeding, storage and anticoagulant on feline CysC was determined. Interestingly, feeding did not affect sCysC concentration, which makes it a potential useful marker for use in practice. However, variability in sCysC between cats was large. Based on our findings, samples can be stored at RT if sCysC is analysed within 24 h, and at 4 °C for 1 week. If samples have to be stored for a longer period, we advise storage at -20 °C and -72 °C for up to 5 months. However, additional studies are required, to value the clinical relevance of our observations. Since we did not investigate the effect of freezing on pCysC, we cannot exclude a different effect of freezing on pCysC compared to sCysC. Until this has been elucidated, we do not advise to compare plasma with serum samples for CysC determination and monitoring.

Acknowledgements

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End Notes

[a] Advia 2120, Siemens, Brussels, Belgium

[b] Architect C16000, Abbott Max-Planck-Ring, Wiesbaden, Germany

[c] Iricell velocity, chemical system and IQ 200 SPRINT, microscopic system; both from Instrumentation Laboratory, Zaventem, Belgium

[d] Immulite 2000 system, Siemens Healthcare Diagnostics, Marburg, Germany

[e] Wask Copan, MLS, Vitek 2 system, BioMerieux, Brussels, Belgium

[f] particle-enhanced nephelometric immunoassay, Siemens Healthcare diagnostics, Marburg, Germany

[g] Vettest analyser, Idexx Laboratories Europe, Amsterdam, the Netherlands

[h] SAS version 9.1, SAS Institute, Cary, IN, USA

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SECTION §4.2

THE EFFECT OF BREED, AGE AND SEX ON FELINE SERUM CYSTATIN C AND ESTABLISHMENT OF A REFERENCE INTERVAL

Liesbeth F.E. Ghys[1], Dominique Paepe[1], Luc Duchateau[2], Elien R.L. Taffin[1], Sofie Marynissen[1], Joris R. Delanghe[3], Sylvie Daminet[1]

[1] Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium

[2] Department of Comparative Physiology and Biometrics, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium

[3] Department of Clinical Chemistry, Microbiology and Immunology, Faculty of Health Medicine and Life Sciences, Ghent University, De Pintelaan 184, B-9000, Ghent, Belgium

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Summary

Chronic kidney disease (CKD) is common in cats, but the routine renal markers, serum creatinine (sCr) and urea are not sensitive or specific enough to detect early CKD. Serum Cystatin C (sCysC) has advantages over sCr, both in humans and dogs and sCysC concentration is significantly higher in cats with CKD than in healthy cats. The objective of this study was to determine the effect of age, sex and breed on feline sCysC and to establish a reference interval for feline sCysC. In total, 130 healthy cats aged 1–16 years were included. Serum CysC was determined with a validated particle-enhanced nephelometric immunoassay. Serum Cr, urea, urine specific gravity (USG), urinary protein:creatinine ratio (UPC) and systolic blood pressure (SBP) were also measured.

No significant differences in sCysC concentration were observed among young, middle-aged and geriatric cats; among female intact, female neutered, male intact and male neutered cats; and among purebred and domestic short- or longhaired cats. The 95% reference interval for feline sCysC was determined to be 0.58–1.95 mg/L. Serum creatinine was significantly higher in geriatric cats than in young cats. Serum urea in geriatric cats was significantly higher than in middle-aged ($P = 0.004$) and young ($P < 0.001$) cats. SBP in geriatric cats was significantly higher than in both middle-aged ($P = 0.004$) and young cats ($P = 0.04$). Male neutered and female neutered cats showed significantly higher serum urea concentrations than female intact cats ($P = 0.003$ and $P = 0.006$, respectively). Male intact cats had a significantly higher UPC than female intact ($P = 0.02$) and female neutered ($P = 0.02$) cats. There were no significant differences among sex groups for USG.

However, it is of concern that sCysC in the majority of cats with CKD in previous studies falls within the RI calculated in this study. Further studies are warranted to evaluate the diagnostic value of sCysC as a renal marker in cats.

Keywords: Cat; Chronic kidney disease; Serum cystatin C; Creatinine; Biological variation; Healthy cat

Introduction

Chronic kidney disease (CKD) is one of the most common feline diseases, with a prevalence of up to 40% in the geriatric cat population.[1] The condition is progressive and the primary cause is mostly unknown,[2] but the ultimate goal in management is early diagnosis and treatment to prevent disease progression.[3-5]

Kidney function can be evaluated by direct tests or indirect markers. Direct tests require measurement of the glomerular filtration rate (GFR), based on clearance of markers that are freely filtered through the glomerulus without tubular reabsorption or secretion.[6] These tests are considered to be the reference standard method for the evaluation of kidney function, but are labour-intensive and time-consuming, and therefore are not routinely used. Indirect markers, such as serum creatinine (sCr) and urea, lack both sensitivity and specificity.[6] Serum Cr in dogs is influenced by diet, hydration status, physical exercise, housing and drugs.[6] Due to these limitations, there is an urgent need for indirect markers that can reliably and easily detect kidney dysfunction.

Cystatin C (CysC) is a low molecular mass (MM), 13 kDa protein.[7] It is a proteinase inhibitor, produced by all nucleated cells and is responsible for the intracellular catabolism of peptides and proteins.[8,9] Cystatin C has most of the properties of an endogenous marker of GFR[10] and several studies in humans[11,12] and dogs[13-15] confirm the superiority of serum CysC (sCysC) to sCr for the detection of renal impairment. Three human assays are available for the measurement of CysC: an enzyme linked immunosorbent assay (ELISA), a particle enhanced turbidimetric assay (PETIA) and a particle enhanced nephelometric assay (PENIA).[16-18] We have recently validated the PENIA for feline CysC determination in serum and urine. We also demonstrated that sCysC and urinary CysC (uCysC) were significantly higher in cats with CKD than in healthy cats.[19]

To use sCysC as a renal marker in practice, it is necessary to establish a reference interval (RI). According to the American Society for Veterinary Clinical Pathology (ASVCP) guidelines, the reference population should represent the animal population for which the RI will be used,[20] and several breeds, ages and both sexes should be included to characterize biological variation and to determine if separate RIs are needed. To the authors' knowledge,

no large-scale studies evaluating sCysC in healthy cats of different ages, sexes and breeds are currently available. However, in dogs there are conflicting accounts of the effect of age and body weight on sCysC.[14,15] The objective of this study was to determine the effect of age, breed and gender on feline sCysC and to establish a RI for feline sCysC.

Materials and methods

Study population

Healthy cats ≥ 1 year of age were recruited. To evaluate the effect of age, cats were divided in three groups: young (1–3 years), middle-aged (3–10 years) and old cats (> 10 years). We aimed to include an equal number of cats in every group. Domestic shorthair (DSH), domestic longhair (DLH) and purebred cats were included with a maximum of 20 cats per breed for purebreds. Cats were considered healthy when there was no history of illness and if no significant abnormalities were detected on physical examination, complete blood cell count (CBC), serum biochemistry profile, including total thyroxin measurement in cats > 6 years, and urinalysis. Cats with both sCr > 141.2 µmol/L and urine specific gravity < 1.035 were excluded. Cats were also excluded if they received non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, antibiotics, β-blockers, angiotensin receptor blockers (ARB) or angiotensin converting enzyme (ACE)-inhibitors within one month before inclusion.

This study was carried out in strict accordance with the recommendations in the Guide for the care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the local and national ethical committees (EC2011_97) and the Deontological Committee of the Belgian Federal Agency for the Safety of the Food Chain (Approval Number EC2011_97). All owners were informed about the study and gave their written informed consent.

Procedures

A standard physical examination was performed on all cats, including systolic blood pressure (SBP) measurement using Doppler ultrasonic technique and a standardised procedure according to the ACVIM consensus guidelines.[21] Cats were considered hypertensive if SBP >

160 mmHg.[21,22] Thyroid gland palpation was performed in cats > 6 years, as previously described.[23,24] All cats were fasted at least 16 h prior to sampling.

Five millilitres of blood were collected by jugular venipuncture using a 23 G needle. Blood samples were then stored at room temperature to allow them to clot prior to centrifugation. After centrifugation (5 min at 2431x *g*), 1 mL of serum was analysed on the day of collection. CBC[a] and serum biochemistry profile[b] were performed in all cats. Serum Cr was determined using a modified Jaffe assay (RI 44.2–141.2 µmol/L). Serum urea was determined by an enzymatic assay (RI 6.16–10.82 mmol/L).

Ten millilitres of urine were collected by cystocentesis using a 22 G needle. Urine specific gravity was determined using a manual refractometer. Urinalysis consisted of a urinary dipstick test,[c] urinary protein:creatinine ratio[d] and urine bacterial culture.[e] Urine was centrifuged (3 minutes at 447 x *g*) and the sediment was analysed within 30 min as previously described.[25]

Serum CysC was determined with the particle enhanced nephelometric immunoassay (PENIA),[f] previously validated for feline CysC determination by our group.[19]

Statistical methods

Analyses were performed with SAS.[g] Serum Cr, urea, sCysC, SBP, USG and UPC levels were compared between purebred and DSH and DLH cats, between young, middle-aged and old cats, and between male, male neutered, female and female neutered cats using a linear fixed effects model with the covariates as categorical fixed effects. Normal probability plots were used as a diagnostic tool and normal distribution was confirmed. A global significance level of 5% was used and Tukey's technique was used to adjust for multiple pairwise comparisons. The 95% RI was determined for all measured parameters with a nonparametric method with the 2.5th and 97.5th fractiles serving as the lower and upper reference limit respectively. Results are shown in the text as mean ± standard deviation.

Results

Study population

One hundred and thirty cats were included: 74 DSH or DLH cats and 56 purebred cats, including British shorthair (*n* = 19), Ragdoll (*n* = 14), Persian (*n* = 8), Sphynx (*n* = 6), Oriental shorthair (*n* = 5), Siamese (*n* = 3) and Birman (*n* = 1). The study population consisted of 85 female cats (40 intact, 45 neutered) and 45 male cats (11 intact, 34 neutered). The mean age of the population was 5.7 years with 48 young, 49 middle-aged and 33 geriatric cats. The mean body weight was 4.1 ± 1.1 kg.

Procedures

In the study population (*n* = 130), mean sCysC was 1.2 ± 0.4 mg/L; mean sCr was 106.2 ± 19.3 µmol/L; mean serum urea was 8.6 ± 1.7 mmol/L; mean USG was 1.038 ± 0.013 and mean UPC was 0.19 ± 0.12. USG was < 1.035 in 25/130 cats. None of these 25 cats had clinical signs of CKD and all had sCr < 141.2 µmol/L. At the time of inclusion, abdominal ultrasonography was performed in two cats and GFR was calculated in three cats based on an exogenous creatinine clearance test. There were no indications of early CKD. Attempts were made to contact the owners of these 25 cats > 2 years after inclusion in the study. Five owners could not be reached. None of the other cats had developed signs of CKD. Two cats died of neoplastic disease, two had run away, three were pregnant and one intact male cat lived abroad. USG and sCr were re-evaluated in 12 cats; all had USG > 1.035 and sCr < 141.2 µmol/L. The descriptive statistics for sCr, urea, sCysC, USG, UPC and SBP of the complete study population are presented in table 1.

Systolic blood pressure (SBP) could be measured according to ACVIM Consensus Guidelines[21] in 104/130 cats. Mean SBP was 135 ± 19 mmHg. Eight cats were hypertensive (range, 160–182 mmHg). The mean age of the hypertensive cats was 6 years and 11 months (range, 2.0–12 years). Systolic blood pressure could be measured in 33/48 young cats, 39/49 middle-aged cats and 32/33 geriatric cats. Two of the young cats, one of the middle-aged cats and five of the geriatric cats were hypertensive.

Descriptive statistics for sCr, urea, sCysC, USG, UPC and SBP are presented in Table 1. No significant differences in sCysC concentration were observed among young, middle-aged and geriatric cats, among female intact, female neutered, male intact and male neutered cats, or among purebred and DSH or DLH cats. Domestic shorthair and DLH cats had significantly higher serum urea concentrations ($P < 0.001$) compared with purebred cats. The geriatric group had significantly higher mean sCr concentration than young cats ($P = 0.03$) and significantly higher serum urea than middle-aged ($P = 0.004$) and young ($P < 0.001$) cats. Geriatric cats also had significantly higher SBP than both middle-aged ($P = 0.004$) and young cats ($P = 0.04$). Male neutered and female neutered cats showed significantly higher serum urea values than female intact cats ($P = 0.003$ and $P = 0.006$, respectively). Male intact cats had a significantly higher UPC than female intact ($P = 0.02$) and female neutered ($P = 0.02$) cats. There were no significant differences among sex, age and breed groups for USG.

The RI for sCysC was 0.58–1.95 mg/L ($n = 130$). The median and 95% RIs for sCr, urea, and sCysC were determined in all 130 cats (Table 2) and also in a subset of cats with USG ≥ 1.035 ($n = 105$). The RI for sCysC was 0.58–1.95 mg/L ($n = 130$) and 0.58–1.97 mg/L ($n = 105$); sCr was 64.5–161.8 μmol/L ($n = 130$) and 64.5–167.0 μmol/L ($n = 105$); and serum urea was 6.2–12.7 mmol/L ($n = 130$) and 6.2 –14.5 mmol/L ($n = 105$). Fig. 1 shows boxplots and 95% RIs for sCysC, sCr and serum urea ($n = 130$).

Table 1. Descriptive statistics for all cats expressed as means (standard deviation). Means sharing the same letter (or no letter) do not differ significantly from each other.

Variable	Age			Sex				Breed	
	Y (*n* = 48)	**M (*n* = 49)**	**G (*n* = 33)**	**FI (*n* = 40)**	**FN (*n* = 45)**	**MI (*n* = 10)**	**MN (*n* = 35)**	**P (*n* = 56)**	**D (*n* =74)**
sCr (µmol/L) (44.2–141.2)	98.6 (23.1)[a]	110.2 (28.4)[a, b]	109.2 (20.2)[b]	98.1 (21.9)	106.6 (24.2)	107.3 (25.1)	112.2 (27.9)	101.1 (25.5)	109.2 (24.2)
Serum urea (mmol/L)	8.0 (1.1)[a]	8.4 (1.4)[a]	9.7 (2.3)[b]	7.9 (1.2)[a]	8.9 (1.8)[b]	8.0 (1.5)[a,b]	9.1 (1.8)[b]	7.7 (1.2)[a]	9.3 (1.8)[b]
sCysC (mg/L)	1.2 (0.4)	1.1 (0.4)	1.1 (0.3)	1.2 (0.4)	1.1 (0.3)	1.2 (0.5)	1.2 (0.4)	1.2 (0.4)	1.1 (0.3)
SBP (mmHg)	133.2 (18.8)[a]	129.3 (16.8)[a]	144.7 (19.4)[b]	133.8 (22.2)	137.8 (17.93)	137.8 (17.5)	133.2 (19.3)	136.2 (17.0)	135.0 (20.3)
USG	1.025 (0.015)	1.047 (0.012)	1.043 (0.011)	1.020 (0.017)	1.045 (0.012)	1.049 (0.012)	1.043 (0.012)	1.046 (0.015)	1.031 (0.012)
UPC	0.2 (0.13)	0.17 (0.07)	0.2 (0.16)	0.17 (0.11)[a]	0.18 (0.14)[a,b]	0.27 (0.14)[c]	0.20 (0.09)[a, c]	0.19 (0.13)	0.19 (0.13)

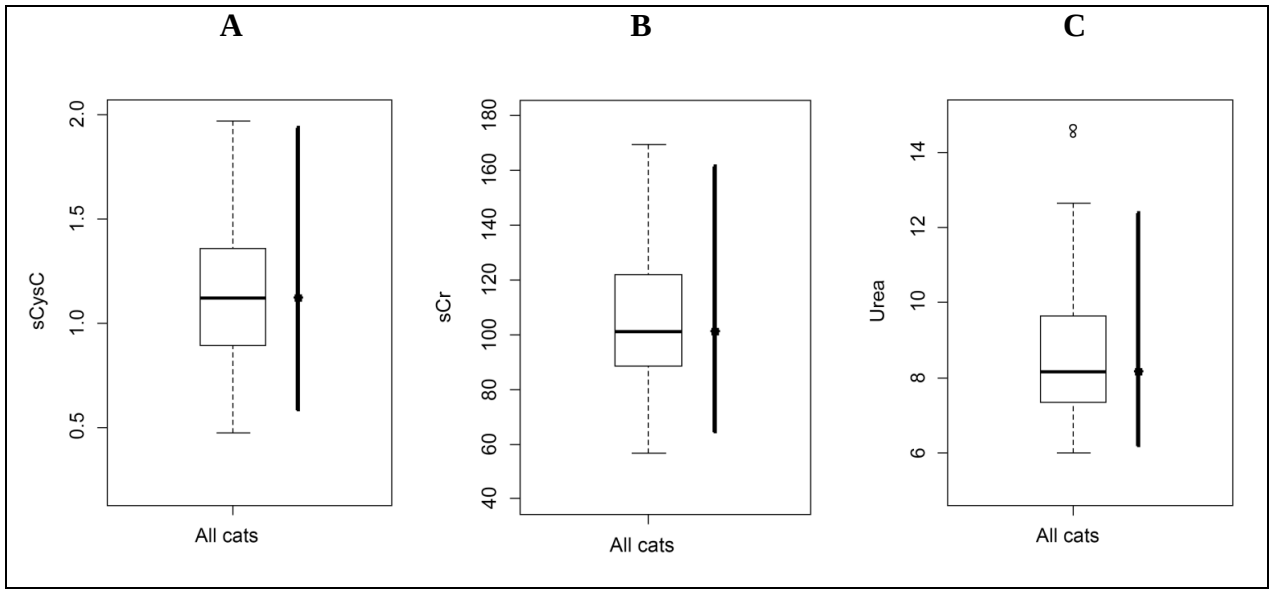
Y, young; M; middle-aged; G, geriatric; FI, female intact; FN, female neutered, MI, male intact; MN, male neutered; P, purebred; D, domestic short-or longhaired cats; sCr, serum creatinine; sCysC, serum Cystatin C; SBP, systolic blood pressure; USG, urine specific gravity; UPC, urinary protein:creatinine ratio.

Table 2. Median (95% reference intervals) for the routine variables measured in 130 healthy cats.

Variable	RI in all cats
sCr (µmol/L)	101.3 (64.5–161.8)
Serum urea (mmol/L)	8.2 (6.2–12.7)
sCysC (mg/L)	1.12 (0.58–1.95)

RI, reference interval; sCr, serum creatinine; sCysC, serum Cystatin C

Figure 1. Boxplot and 95% reference interval for serum cystatin C (sCysC) (A); serum creatinine (sCr) (B); serum urea (C).



Discussion

This is the first article to determine RIs for feline sCysC measured using PENIA according to the ASVCP Guidelines[20] and is one the largest study of the effects of age, breed and sex on feline sCysC in healthy cats. We did not observe a significant difference in sCysC concentration among young, middle-aged and geriatric cats; male intact, male neutered, female intact and female neutered cats; or among DSH, DLH and purebred cats. Additionally, no effect of age, sex and bodyweight was demonstrated in a previous study.[26] Our findings support the need for further clinical investigation of feline sCysC.

Surprisingly, our observations were in contrast with human and some canine reports. In humans, it has been shown that sCysC is higher in males and older people.[27,28] and differences among races have been described.[27] In dogs, lower sCysC concentrations were observed in younger animals and in those weighing < 15 kg,[14] but these findings have been contradicted by other studies.[15,29] Moreover, significant differences between male and female dogs were not observed.[15] Since we did not observe any effects of age, sex and breed on sCysC, partitioning of the RI is not needed for these variables. The low biological variation is a major advantage of sCysC over sCr.

In our study, purebred cats did not have a significantly higher sCr or sCysC concentration than DSH or DLH cats. However, sCr concentrations are influenced by breed in dogs[30-32] and by race in humans.[33] This could be due to differences in muscle mass,[6] and also to differences in GFR. The effect of race on GFR in humans[34] and breed and bodyweight in dogs[35-37] has been demonstrated. To the authors' knowledge, the effect of breed on GFR in cats has not been investigated. However, one group described a potential breed effect on feline sCr, as Birman cats appeared to have higher sCr concentrations than the RI determined in specific pathogen free adult cats.[38] A significant effect of breed on sCr was observed for Birman, Maine Coon, Chartreux and Persian cats, and separate RIs were proposed.[39] The need for breed-specific RIs for sCr in Birmans and Siberian cats has recently been confirmed.[40]A preliminary retrospective study by our group, hypothesized that a breed-specific RI would be needed for sCr in Ragdolls,[41] but the hypothesis was rejected in a later prospective study in which sCr concentrations in Ragdolls were similar to control cats.[42] Larger studies in more breeds are needed to investigate possible breed differences in feline sCr concentrations.[20]

Previous studies reported higher sCr concentrations in cats with increasing age.[43,44] We also demonstrated that geriatric cats had significantly higher sCr concentrations than young cats. This is surprising, since we expected lower sCr concentrations due to decreasing muscle mass in older cats.[6,44] A significant correlation between sCr and body weight was observed in a population of DSH/DLH cats, but the authors attributed this to increased adiposity. However, no body fat content was measured.[45] No effect of age on sCr was observed in that population of DSH/DLH cats.[45] Although we found a significant difference, the difference in sCr between the three age groups in our study was small and an obvious overlap could be observed, so the difference is probably not clinically relevant. However, we cannot exclude that some of the older cats had early CKD explaining the higher sCr in old cats. No differences among female intact, female neutered, male intact and male neutered cats were observed in the present study.

We also found significant differences among groups for other renal parameters. For urea, several significant differences were observed, namely, between middle-aged and geriatric cats, geriatric and young cats, purebred and DSH or DLH, female intact and female neutered and between female intact and male neutered cats. Urea is more likely to be influenced by non-renal factors than sCr.[6,46] In humans, ethnical differences have been observed.[47] Paltrinieri et al.[40] demonstrated the need for a breed-specific RI for urea in cats. We are not aware of other reports about the effects of age and sex on feline serum urea. We also observed significantly higher UPC in male intact cats than in female intact and female neutered cats. However, there was no significant difference in UPC when male intact were compared with male neutered cats. This finding contrasts with other studies, in which higher UPC was assumed for intact male cats, due to higher urinary cauxin concentrations. Cauxin is a protein that regulates the production of felinine, a pheromone precursor.[48-50]

Geriatric cats had a significantly higher SBP than the middle-aged and young cats. An age-effect on SBP has previously been described in healthy cats, [25,51] but other studies did not observe a significant association between age and SBP.[52,53] Single measurements, such as those used in our study, are not sufficient for the diagnosis of hypertension.[21] Hypertension should be confirmed using multiple measurements and supported with a diagnostic workup to establish the underlying cause,[21] but this was beyond the scope of our study.[54,55] Although we cannot rule out hypertension merely due to 'white coat effect', most of the hypertensive cats in our study were in the geriatric group and it is possible that other causes were responsible

for increased SBP in this group.[54,55] Nevertheless, longitudinal studies evaluating SBP, renal and thyroid function in aging cats are necessary to determine if hypertension is independently related to age or to co-existing diseases such as declining kidney function or hyperthyroidism. Interestingly, significantly higher SBP values have been reported in female cats,[52] and in humans,[56] but this sex predilection was not apparent in our study.

The second major objective of this study was to determine a RI for feline sCysC (0.58–1.95 mg/L). Since no effect of age, gender and breed was apparent, results from the entire population of enrolled cats were included in the RI calculations. The ASVCP guidelines,[20] state that a minimum sample size of 120 animals is necessary for RIs to be established. We used the recommended nonparametric method, with the 2.5th and 97.5th percentiles serving as lower and upper reference limits, respectively.[20] Our RI does not correspond with the RI (0.34–4.11 mg/L) proposed by another group,[57] but there are important differences between the two studies. Firstly, Martin et al.[57] measured plasma CysC rather than sCysC using a turbidimetric assay, but at the time of the present study, validation reports or comparisons with the PENIA assay for feline sCysC were not available for this method. Secondly, clinical data from the cats in the Martin et al. study[57] were unreported so concurrent diseases that might have affected sCysC cannot be excluded. Finally, Martin et al.[57] calculated a RI for plasma CysC based on a sample set of 99 cats, rather than the minimum 120 cats required by the ASVCP Guidelines.[20]

Previous studies have used PENIA to measure sCysC concentrations in a small number of healthy cats, reporting values that fell within the RI of this study.[19,26] However, in those studies, sCysC concentrations in some cats fell within RI calculated in our study (7/10 cats with CKD,[19] 46/46 cats with CKD).[26] It is possible that CysC is not a reliable marker for the early detection of kidney dysfunction in cats. However, our study is inconclusive on this point and this subject requires further investigation.

There are only two published studies reporting the evaluation of feline sCysC and GFR.[26,58] Serum CysC was not better correlated with GFR than with sCr in cats with hyperthyroidism and no change in sCysC was observed when before- and after-treatment values were compared.[58] However, in healthy cats and cats with CKD, a remarkably better correlation with GFR was found for sCysC compared to sCr.[26] Because GFR was not measured in our study, we cannot be certain that some cats of our study population did not

have early kidney impairment. Further research on feline sCr and sCysC is needed to investigate potential correlations with GFR. If the potential use of sCysC as a marker of GFR in clinical practice is to be explored, its ability to detect early kidney dysfunction should be determined by long-term monitoring of apparently healthy and also by using cats with medical conditions that might affect CysC production. Human studies have demonstrated that sCysC results in underestimation of GFR in patients with hyperthyroidism,[59] due to a stimulatory effect of thyroid hormones on CysC production.[60]

The sCr concentration of seven cats in our study population exceeded the upper limit of RI for sCr established by our laboratory (44.2–141.2 μmol/L). All cats with sCr > RI had a USG > 1.035. A previous study by our group reported that the RI from the external laboratory was not appropriate for broad interpretation of feline sCr,[25] since it was calculated using sCr concentrations from cats aged 6 months to 1 year. Inappropriate RIs have also been reported for other laboratories.[61] Because the development of laboratory-specific RIs is recommended,[20] we also calculated a RI for sCr (64.5–161.8 μmol/L). It is valuable to compare our results with sCr concentrations measured using the same assay method. None of the cats in our study had a sCr concentration over the upper reference limit (177 μmol/L) of a previously published RI for feline sCr,[62] measured using a modified Jaffe reaction.

Twenty-five cats in our study had USG < 1.035 and 2/25 had isosthenuric urine. However, none of those cats had sCr concentrations exceeding the upper RI calculated at our laboratory. It has been shown that USG shows daily variation[63] and therefore, low USG does not necessarily reflect impaired kidney function.[64] More than 2 years after inclusion in this study, the owners of 20 cats were contacted again. None of the cats had developed clinical signs of CKD and all cats that were re- evaluated had USG > 1.035 and sCr < 141.2 μmol/L. To the authors' knowledge, there are no published studies reporting the evaluation of USG in a large healthy cat population. In a prospective study of healthy Ragdoll cats, 8/62 healthy control cats aged 2.7 ± 1.6 years had USG < 1.035.[42] It has been hypothesised that the normal range for feline USG is 1.001–1.065[65] and even up to 1.080.[66]

Nevertheless, studies evaluating USG in a large healthy cat population of different ages and breeds are warranted. Additionally, our study determined that the RIs of sCysC, sCr and serum urea in a subset of 105 cats with USG ≥ 1.035 were minimally different from RIs for these parameters from the entire study population (n = 130). However, the upper

reference limits for these parameters were higher in the 105-cat group, since all 25 cats with low USG had low sCr and low serum urea concentrations.

This study is limited by the large proportion of DSH and DLH cats enrolled vs. purebred cats, which might not necessarily represent the cat population examined by veterinarians. A second limitation is the lack of GFR measurement and limited follow-up information. Based on sCr concentrations and USG measurements only, we do not know if some cats might have had early kidney impairment. Finally, while each of the subgroups contained a reasonable number of cats, larger subgroups would generate more reliable data for comparisons of renal parameters.

Conclusion

To our knowledge, this study is the first report about the effect of age, breed and gender on feline sCysC concentration and the establishment of a RI in the more than the required 120 healthy cats. A promising result is that there was no biological variation observed for sCysC compared to sCr. Now, further studies are necessary to evaluate if sCysC is more sensitive and specific to detect a decreased GFR. Healthy cats and cats with CKD need to be studied and their GFR needs to be correlated with their sCysC concentration.

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End Notes

[a] Advia 2120, Siemens, Brussels, Belgium

[b] Architect C16000, Abbott Max-Planck-Ring, Wiesbaden, Germany

[c] Iricell velocity, chemical system and IQ 200 SPRINT, microscopic system; both from Instrumentation Laboratory, Zaventem, Belgium

[d] Immulite 2000 system, Siemens Healthcare Diagnostics, Marburg, Germany

[e] Wask Copan, MLS, Vitek 2 system, BioMerieux, Brussels, Belgium

[f] Behring Nephelometer (BN) ProSpec, Siemens Healthcare Diagnostics, Marburg, Germany (SAS version 9.3, SAS Institute Inc.)

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CHAPTER V

CLINICAL VALIDATION OF FELINE CYSTATIN C

SECTION §5.1

SERUM AND URINARY CYSTATIN C IN CATS WITH FELINE IMMUNODEFICIENCY VIRUS INFECTION AND CATS WITH HYPERTHYROIDISM

Liesbeth F.E. Ghys[1], Dominique Paepe[1], Elien R.L. Taffin[1], Eva Vandermeulen[2], Luc Duchateau[3], Joris R. Delanghe[4], Pascale Smets[1], Sylvie Daminet[1]

[1] Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium

[2] Department of Medical Imaging and Orthopaedics of Small Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium

[3] Department of Physiology and Biometrics, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium

[4] Department of Clinical Chemistry, Microbiology and Immunology, Faculty of Health Medicine and Life Sciences, Ghent University, De Pintelaan 184, B-9000, Ghent, Belgium

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Summary

Cystatin C (CysC) is a marker for glomerular filtration rate and tubular dysfunction. A pilot study has shown that cats with chronic kidney disease (CKD) have a significantly higher serum CysC (sCysC) and urinary CysC (uCysC) concentration compared with healthy cats. Hyperthyroidism may lead to kidney dysfunction and renal abnormalities are also common in cats with feline immunodeficiency virus (FIV). Higher sCysC concentrations have been observed in humans with human immunodeficiency virus or hyperthyroidism.
The objective of this study was to investigate sCysC and uCysC in cats with hyperthyroidism and cats with FIV.

Thirty cats with FIV, 26 hyperthyroid cats and 28 healthy cats were included. Serum CysC and uCysC:creatinine ratio (uCysC/uCr) were measured with a human particle enhanced nephelometric immunoassay (PENIA), previously validated for feline CysC measurement. Routine renal variables (serum creatinine (sCr), urine specific gravity (USG), urinary protein:creatinine ratio (UPC)) were also measured in the three groups.

Cats with hyperthyroidism had significantly higher sCysC, lower sCr concentrations higher uCysC/uCr and higher UPC than healthy cats. Cats with FIV-infection did not show significantly higher sCysC concentration, but had a significantly higher sCr and UPC than healthy cats. Urinary CysC could be detected in only four of them.

This study demonstrated that sCysC is increased in cats with hyperthyroidism, in contrast with sCr, but not in cats with FIV. Many hyperthyroid cats, but only four cats with FIV had elevated uCysC/uCr. Further studies may reveal if uCysC might be a valuable marker for tubular dysfunction in cats.

Keywords: Feline; Creatinine; Renal variable; Glomerular filtration rate; Tubular dysfunction

Introduction

Cystatin C (CysC) is a low molecular mass (MM) protein, responsible for the intracellular catabolism of peptides and proteins.[1,2] Most of the properties for endogenous glomerular filtrate rate (GFR) markers apply for CysC.[3] Studies in humans[4,5] and dogs[6-8] have shown the superiority of serum CysC (sCysC) compared to serum creatinine (sCr) in the early detection of renal impairment. Furthermore, urinary CysC (uCysC) was used as a tubular marker in both human[9,10] and canine[11] studies. Therefore, interest in investigating CysC as an early renal marker in cats has increased, as chronic kidney disease (CKD) is one of the most common diseases in older cats, with a prevalence of up to 30% in cats older than 15 years.[12] Our group has recently validated a human particle enhanced nephelometric immunoassay (PENIA) for feline CysC measurement and has demonstrated a significant difference in sCysC and uCysC concentration between healthy cats and cats with CKD.[13] We also reported that the biological factors age, breed and sex do not influence sCysC, in contrast to sCr and serum urea.[14] The obtained reference interval (RI) for sCysC was (0.58–1.95 mg/L).[14]

Chronic kidney disease mainly affects aged cats.[15] In this population, hyperthyroidism is also a common disease with a prevalence of up to 11%.[16] Furthermore, a recent study of our group could demonstrate a feline immunodeficiency virus (FIV) seroprevalence of 14% in cats older than 6 years.[17] This means that concurrent CKD and FIV-infection or hyperthyroidism might occur. In addition, both hyperthyroidism and FIV appear to have a negative effect on kidney function.[18,19]

Hyperthyroidism causes an increased metabolism, which is associated with decreased vascular resistance, increased cardiac output, increased renal blood flow, increased GFR and hypertrophic and hyperplastic tubuli. Untreated hyperthyroidism can induce renal damage or exacerbate existing renal disease.[20] Due to increased GFR, but also due to decreased muscle mass in hyperthyroid patients, sCr decreases, which leads to an overestimation of renal function.[21] The effect of thyroid function on sCysC has been investigated in human medicine. After treatment for hyperthyroidism, sCysC decreases over time.[21] This means GFR is underestimated when considering sCysC levels in untreated hyperthyroid patients, in contrast to sCr.

Human patients with human immunodeficiency virus (HIV) infection have a higher risk of developing CKD.[22,23] Therefore, interest raised to use sCysC as a renal marker in HIV-positive patients and it has been shown that sCysC is higher in patients with HIV than healthy controls.[24,25] There appears to be an association between FIV and kidney abnormalities,[18,26] but the effect of FIV on sCysC and uCysC has not been studied.

The objective of this study was to evaluate sCysC and uCysC in cats with hyperthyroidism and in cats naturally infected with FIV.

Materials and methods

Study population

Healthy cats, FIV-positive cats and hyperthyroid cats were prospectively recruited. Cats were considered healthy when there was no disease history, and if no clinically relevant abnormalities were detected upon physical examination, complete blood cell count (CBC), serum biochemistry profile and urinalysis.[12] Healthy cats were excluded when they had received medication within one month prior to inclusion that might influence renal function, such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, antibiotics, β-blocking agents, angiotensin receptor blockers (ARB) or angiotensin converting enzyme (ACE)-inhibitors.

Feline immunodeficiency virus infection was confirmed with a positive in-house FIV test.[a] Owing to ethical considerations, FIV-infected cats treated with NSAIDs and/or antibiotics within one month prior to inclusion were not excluded. Cats were not included when they showed abnormalities compatible with a concurrent disease on physical exam, CBC, serum biochemistry profile and/or urinalysis.

Cats with hyperthyroidism were included when they demonstrated clinical signs compatible with hyperthyroidism, increased total thyroxine (TT4) concentration and increased thyroidal uptake of pertechnetate ($^{99m}TcO_4^-$) on a diagnostic scintigraphic scan. Antithyroid drugs had to be stopped at least two weeks before inclusion, since all included hyperthyroid cats were treated with ^{131}I after the sampling and it has been shown that concurrent administration of anti-thyroid drugs increases the risk of iatrogenic hypothyroidism and adversely affects the effective half-life of ^{131}I.[27,28] Methimazole was stopped for only three days in cats previously treated with $\geq$ 10 mg methimazole per day, to avoid the risk of thyroid storm by two weeks discontinuation of anti-thyroid drugs.[29] Cats were excluded if there was evidence of a concurrent disease on physical exam, CBC, serum biochemistry profile or urinalysis.

In all three groups, cats with isosthenuric urine i.e. USG < 1.015 were excluded. Cats with USG < 1.035 were only included if concurrent azotemia i.e. sCr > 161.8 µmol/L was absent. It is known that low USG without concurrent azotemia does not necessarily reflect

impaired kidney function.[30] In all FIV-positive cats and healthy cats TT4 was measured, and FIV was tested in all hyperthyroid and healthy cats. Healthy cats and FIV-positive cats were excluded if TT4 > 45.15 nmol/L. In addition, hyperthyroid and healthy cats that tested FIV-positive were also excluded.

Analytical methods

A standard physical examination was performed in all cats, including thyroid gland palpation in the FIV-infected cats suspected of hyperthyroidism and all healthy cats older than six years. An in-house FIV test[a] was performed in all cats following the test guidelines.

Five mL of blood was taken by jugular venepuncture using a 23 G needle. One mL was collected in an EDTA-containing tube for determination of complete blood cell count in all cats.[b] After centrifugation (5 mins at 1931 x *g*), 1 mL of serum was analysed the same day. Serum biochemistry[c] was performed in all cats. Serum Cr was determined with a modified Jaffe assay with a RI of (64.5–161.8 µmol/L), previously determined by our group according to the American Society of Veterinary Clinical Pathology (ASVCP) guidelines.[14,31]

Total thyroxine was measured in all cats with a chemiluminescent immunoassay,[d] previously validated in cats (RI (14.19–45.15 nmol/L)).[32] In all cats, 10 mL of urine was taken by cystocentesis with a 22 G needle. Urine specific gravity was determined with a manual refractometer. Urinalysis consisted of a urinary dipstick test,[e] measurement of the urinary protein:creatinine ratio (UPC),[f] sediment analysis and bacterial culture.[g] Urine was centrifuged (3 min at 365 x *g*) and urinary sediment was analysed within 30 min according to Paepe et al.[17] One mL of serum and 1 mL of the urinary supernatant was frozen at -72 °C until batched analysis.

Serum CysC and uCysC were determined with the human PENIA.[h] This assay is based on the dispersion of light caused by immune complex formed by CysC and latex particles coated with polyclonal anti-human CysC antibodies. The human PENIA has recently been validated for feline CysC determination both in serum and urine.[13]

Statistical analysis

Analyses were performed with statistical software.[i] The uCysC concentration was below the detection limit (LOD) in several cats and TT4 concentration was higher than the upper LOD in several hyperthyroid cats. Therefore, nonparametric techniques were used and all measurements for uCysC below the LOD were set at 0.0464 mg/L and all measurements for TT4 above the LOD were set at 193.5 nmol/L. The non-parametric Wilcoxon rank sum test was used to compare sCysC and uCysC/uCr ratio, sCr, UPC, USG between cats with hyperthyroidism or FIV and healthy cats. Spearman rank correlation coefficients were calculated between sCysC and sCr, between sCysC and uCysC/uCr and between UPC and uCysC/uCr in the cats with hyperthyroidism, cats with FIV and healthy cats; between sCysC, sCr and TT4 in the cats with hyperthyroidism and healthy cats. The level of significance was set at 0.05.

Results

Study population

Ninety cats (30 hyperthyroid, 30 FIV-infected and 30 healthy) were evaluated, but only 84 cats were included, since four of the hyperthyroid cats and two healthy cats tested FIV-positive. Their signalment is presented in Table 1. Within one month prior to inclusion, antibiotics were administered in 10 FIV-cats, NSAIDs in two FIV-cats and eight FIV-cats had received both. At the time of inclusion, six of them still received antibiotics, one received NSAIDs and one received both. Two of the hyperthyroid cats were not treated with anti-thyroid drugs before inclusion. In 23 cats, therapy was ceased two weeks before inclusion. In one cat, anti-thyroid therapy was ceased three days before inclusion.

Table 1. Signalment of all recruited cats. Age and body weight are presented as mean ± standard deviation.

Cat population / Variable	**Cats with FIV (*n* = 30)**	**Cats with HT (*n* = 26)**	**Healthy Cats (*n* = 28)**
Breed	29 DSH/DLH, 1 Maine Coon	25 DSH/DLH, 1 Norwegian Forest cat	25 DSH/DLH, 1 Ragdoll, 2 Peterbalds
Gender	3 M, 24 MN, 1 F, 2 FN	4 M, 10 MN, 2 F, 10 FN	4 MN, 2 F, 22 FN
Age	6.7 ± 2.8 years	13.3 ± 2.0 years	10.5 ± 3.7 years
Body weight	4.5 ± 1.0 kg	3.8 ± 0.9 kg	4.0 ± 0.9 kg

FIV, feline immunodeficiency virus; HT, hyperthyroidism; DSH/DLH, domestic short-or longhair cats; M, male; MN, male neutered; F, female; FN, female neutered.

Analytical procedures

Serum CysC, uCysC/uCr, the routine renal variables (sCr, USG, UPC) and TT4 were measured in all cats. Ten hyperthyroid cats had a TT4 concentration above 193 nmol/L, the upper measurable concentration limit of the analytical device.[c]

The descriptive statistics for the variables sCr, USG, UPC, sCysC and uCysC/uCr for the FIV, hyperthyroid and healthy cats are presented as median (range) in Tables 2 and 3. Several cats had USG < 1.035 without concurrent azotemia: 5/30 FIV-positive cats, 9/26 hyperthyroid cats and 6/28 healthy cats. For the healthy cats, there were no indications of early CKD at the time of inclusion since GFR was normal based on a combined exogenous Cr-iohexol clearance test in four of them and abdominal ultrasound revealed no renal abnormalities in one cat. More than two years after inclusion, USG was > 1.035 without concurrent azotemia in the other healthy cat. For the five FIV-cats no follow-up data were available. For the hyperthyroid cats, follow-up data after ^{131}I-treatment were only available for three cats and none of them developed azotemia when they became euthyroid. Three of the six other cats were treated prior to sampling with antithyroid drugs and treatment was stopped two weeks before sampling. In these three cats, sCr, TT4 and urea remained normal during medical treatment for hyperthyroidism, making concurrent CKD in these cats less likely.

Table 2. Descriptive statistics for the variables of the included cats with FIV, hyperthyroidism and healthy cats. All variables are expressed as median (range).
* indicates if the variable is significantly different compared with the healthy cats.

Cat population / Variable	**Cats with FIV (*n* = 30)**	**Cats with HT (*n* = 26)**	**Healthy cats (*n* =28)**
sCr (µmol/L)	92.4 (67.2–146.7)*****	69.8 (38–109)*****	99.9 (64.5–142.3)
USG	1.044 (1.020–1.065)	1.039 (1.015–1.060)	1.047 (1.020–1.060)
UPC	0.21 (0.07–0.87)*****	0.52 (0.17–1.14)*****	0.18 (0.11–0.37)
sCysC (mg/L)	0.8 (0.3–1.7)	1.1 (0.4–1.8)*****	0.8 (0.4–1.2)
uCysC/uCr (mg/mol)	< LOD/uCr ***** (< LOD/uCr–118.4)	21.1***** (< LOD/uCr –618.5)	< LOD/uCr

FIV, feline immunodeficiency virus; HT, hyperthyroidism; sCr, serum creatinine; USG, urine specific gravity; UPC, urinary protein:creatinine ratio; sCysC, serum cystatin C; uCysC/uCr, urinary cystatin C:creatinine ratio; LOD, limit of detection, 0.0464 mg/L.

Table 3. Routine renal variables of all included cats, with the number of cats having values below, within and above the reference interval.

Cat population / Variable	Cats with FIV (*n* = 30)	Cats with HT (*n* = 26)	Healthy cats (*n* = 28)
sCr (µmol/L)			
< 64.5	0	1	0
64.5–161.8	30	25	28
> 161.8	0[a]	0[a]	0[a]
Serum urea (mmol/L)			
< 6.16	4	3	0
6.16–10.82	23	17	23
> 10.82	3	6	5
USG			
< 1.015	0[a]	0[a]	0[a]
1.015–1.035	7	9	5
> 1.035	23	17	23
UPC			
< 0.2	15	2	16
0.2–0.4	11	8	12
> 0.4	4	16	0[a]

FIV, feline immunodeficiency virus; HT, hyperthyroidism; sCr, serum creatinine; USG, urine specific gravity; UPC, urine protein:creatinine ratio. [a] Consequence of the inclusion criteria: healthy cats could not have UPC > 0.4; USG had to be > 1.015 and sCr < 161.8 µmol/L in all cats.

Cats with hyperthyroidism had significantly higher sCysC (P = 0.01), higher uCysC/uCr ratio (P < 0.001), lower sCr (P < 0.001) and higher UPC (P < 0.001) than healthy cats. Urinary CysC/uCr was significantly higher in FIV-positive cats compared with healthy cats (P < 0.001). However, only four of the FIV-positive cats had a detectable uCysC concentration. One of them had not received NSAIDs or antibiotics prior to sampling. The other three had received both NSAIDs and antibiotics within two weeks prior to sampling but did not receive medication at the time of blood collection. In addition, a significantly higher sCr concentration (P < 0.001), a higher UPC (P < 0.001), but no significantly higher sCysC concentration was present compared with the healthy cats. The boxplots are presented in Figure 1 and 2.

Figure 1. Box-plot of serum cystatin C (sCysC) (mg/L) for cats with hyperthyroidism (HyperT4; *n* = 26); feline immunodeficiency virus (FIV; *n* = 30) and healthy cats (healthy: *n* = 28).

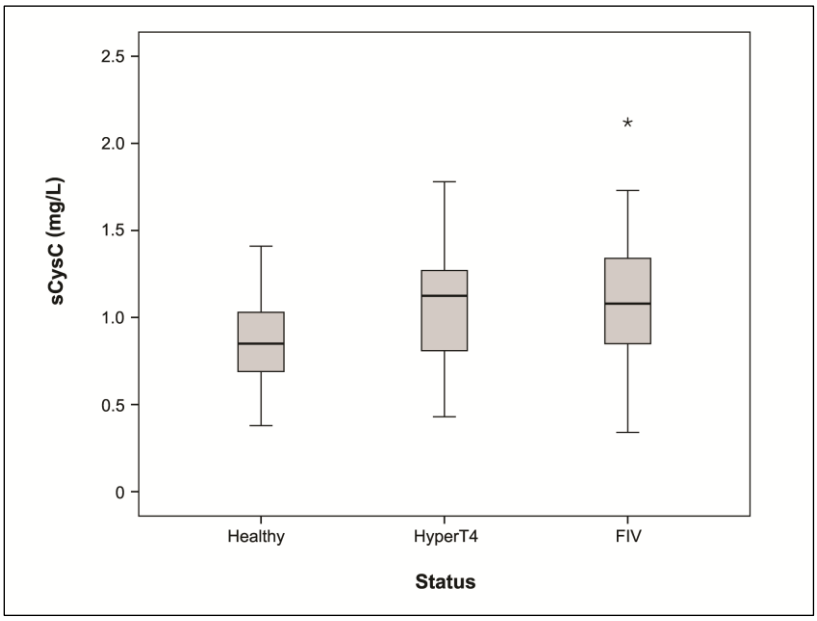
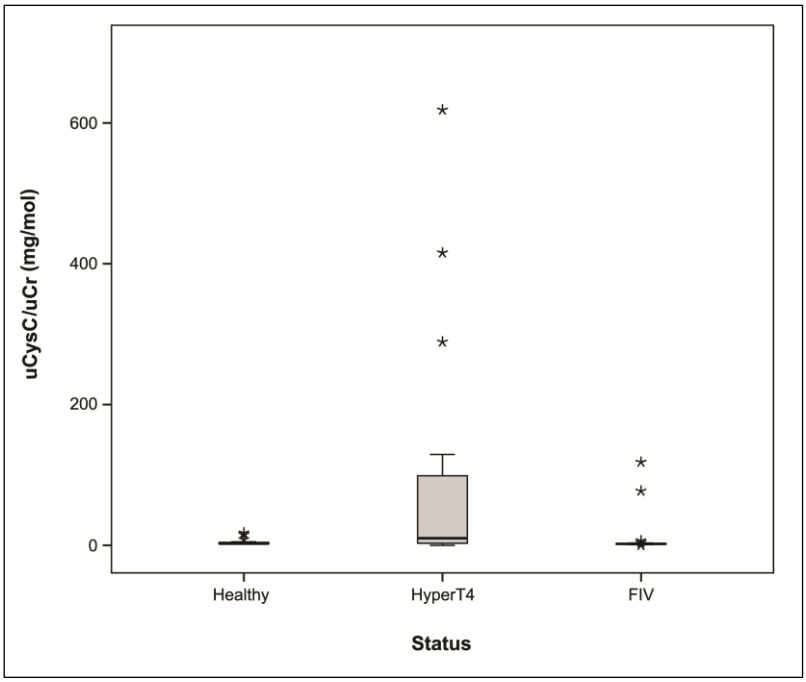


Fig 2. Box-plot of the urinary cystatin C:creatinine ratio (uCysC/uCr ratio) for cats with hyperthyroidism (HyperT4; *n* = 26); feline immunodeficiency virus (FIV; *n* = 30) and healthy cats (healthy: *n* = 28).



The correlation coefficients are presented in Table 4. Only in the hyperthyroid cats a significant correlation was observed between UPC and uCysC/uCr ($P < 0.001$) and between sCysC and uCysC ($P = 0.03$). A positive, but no significant correlation was found between sCysC and TT4 in the hyperthyroid cats. In contrast, sCr was negatively and significantly correlated with TT4 ($P = 0.003$). In the healthy cats however, a negative but no significant correlation with TT4 was observed for both sCr and sCysC. No significant correlation between sCysC and sCr was found in the three groups.

According to our established RI of (0.58–1.95 mg/L),[14] none of the cats had a sCysC concentration above the upper reference limit. Two cats with FIV, one cat with hyperthyroidism and four healthy cats had a sCysC concentration below the RI. All healthy cats, 26 cats with FIV and eight cats with hyperthyroidism had uCysC/uCr below detection limit.

Table 4. Spearman rank correlations for sCysC, sCr, uCysC/uCr, TT4 and UPC ratio in cats with FIV, hyperthyroid cats and healthy cats.

Variable 1	Variable 2	Condition	Correlation coefficient	*P*
sCysC	**sCr**	Hyperthyroidism FIV Healthy	-0.20 -0.05 -0.12	0.32 0.81 0.54
sCysC	**uCysC/uCr**	Hyperthyroidism FIV Healthy	0.42 0.07 0.26	0.03 0.73 0.18
UPC	**uCysC/uCr**	Hyperthyroidism FIV Healthy	0.71 0.27 0.16	< 0.001 0.15 0.42
sCysC	**TT4**	Hyperthyroidism Healthy	0.24 -0.18	0.33 0.43
sCr	**TT4**	Hyperthyroidism Healthy	-0.55 -0.09	0.003 0.71

FIV, feline immunodeficiency virus; sCysC; serum cystatin C; uCysC/uCr, urinary cystatin C:creatinine ratio; TT4, serum total thyroxine; sCr, serum creatinine; UPC, urinary protein:creatinine ratio.

Discussion

Feline sCysC and uCysC were evaluated in cats with hyperthyroidism and in cats with FIV. Cats with hyperthyroidism had significantly higher sCysC and uCysC/uCr compared with healthy cats. However, only few FIV-infected cats had detectable uCysC.

For the hyperthyroid group, routine renal variables, with the exception of USG, significantly differed from healthy cats. Thyroid dysfunction has an impact on the kidney function, by influencing renal blood flow, GFR, tubular secretion and absorption, electrolyte pumps and kidney structure.[19] Owing to increased GFR, but also to decreased muscle mass in patients with hyperthyroidism, sCr decreases, masking possible underlying CKD.[21] Opposite effects are observed in patients with hypothyroidism.[33,34] In our study, sCr was indeed significantly lower and UPC significantly higher in the hyperthyroid cats compared to the control group. Our findings are in accordance with previous studies evaluating kidney function in hyperthyroid cats.[35-37]

The hyperthyroid cats had a significantly higher sCysC concentration than the healthy cats, in contrast to sCr which was significantly lower. Based on our established RI for sCysC of 0.58–1.95 mg/L,[14] hyperthyroid cats cannot be distinguished from healthy cats. There was no significant correlation between sCysC and TT4, and an overlap in sCysC concentration between healthy cats and cats with hyperthyroidism was observed (Figure 1). Our observations are comparable with human studies.[21,34,38] In humans, GFR is underestimated in hyperthyroid patients when considering sCysC concentration[39] and the same might be true in cats. The exact mechanism for a higher sCysC concentration in hyperthyroidism is not yet revealed. Nevertheless, several hypotheses have been proposed.[21,40,41] Triiodothyronine (T3) should increase the level of transforming growth factor-β1 (TGF-β1), which might in turn stimulate the secretion of sCysC.[41] This hypothesis appears the most plausible, as stimulation of CysC secretion by TGF-β1 in vascular smooth muscle cells has been demonstrated.[42,43]

To our knowledge, this is the first study comparing sCysC between healthy cats and cats with hyperthyroidism. In an abstract by Jepson et al.,[44] sCysC was measured in 19 hyperthyroid cats before and after treatment with ^{131}I. In contrast with human data,[45] no significant change in sCysC concentration was observed while a significant change was

reported in the GFR and sCr concentration. The authors did not find a correlation between GFR and sCysC in the hyperthyroid cats before treatment. This study by Jepson et al. and our findings suggest that sCysC might not be a reliable GFR marker in cats with hyperthyroidism, which is comparable with the findings in human medicine.[46] However, additional studies with GFR measurement and comparison with healthy cats are warranted.

Hyperthyroid cats had significantly higher uCysC/uCr than the control group. However, not all hyperthyroid cats had a detectable uCysC concentration. Moreover, the correlation coefficient between UPC and uCysC/uCr was significant (Table 4). The cats without a detectable uCysC concentration, did not have proteinuria, while all hyperthyroid cats with UPC > 0.4 did have a detectable uCysC concentration. However, we observed a significant correlation between sCysC and uCysC/uCr, which suggests that uCysC is not independent from sCysC. Further studies will reveal if uCysC is a good marker for proximal tubular damage in hyperthyroid cats. Other tubular markers, such as urinary N-acetyl-β-D-glucosaminidase (uNAG)[36] and urinary retinol-binding protein (uRBP),[35] do fulfil the required properties to detect tubular damage in hyperthyroid cats.

Cats with FIV had significantly higher uCysC/uCr but no significantly higher sCysC concentration. However, only 4/30 cats had a detectable uCysC concentration, and three of them had received both NSAIDs and antibiotics within two weeks prior to blood collection which could have influenced the results. However, uCysC could not be detected in all FIV-positive cats that had received medication prior to sampling. Further studies are required to investigate if presence of uCysC is due to FIV or administration of NSAIDs. In contrast to the hyperthyroid cats, there was no significant correlation between uCysC/uCr and UPC, since not all of the proteinuric cats had a detectable uCysC concentration. None of our cats with FIV had a sCr concentration above the upper reference limit (161.8 µmol/L) of the RI we established in a recent study.[14] Nevertheless, it has been described that cats with FIV can have tubular abnormalities, without concurrent azotemia[47] and several studies describe an association between proteinuria and FIV-infection.[18,48] Our study suggests that tubular dysfunction is absent or not severe enough in most FIV cats to cease tubular catabolism of CysC, leading to CysC appearance in the urine. The presence of CysC in urine of HIV-infected persons has only been reported in patients receiving a combination antiretroviral therapy (cART),[49] but not in non-treated HIV-patients. Serum CysC was not significantly different between healthy cats and cats with FIV, even though sCr was significantly higher in

the FIV cats. A higher prevalence of azotemia in FIV-infected cats has been described,[48,50] but other reports claim no association between FIV and renal azotemia.[18,51] Our results are in contrast with human reports, where a significantly higher sCysC concentration was observed in HIV-infected patients.[25,52] In addition, a correlation of HIV-RNA and $CD4^+$ T-cell count with a decreased sCysC concentration after therapy has been demonstrated,[25] suggestive for an association of HIV-replication with the sCysC level,[53] and an underestimation of GFR in non-treated HIV-patients. However, in no human report, GFR was measured by a gold standard method, so it is unclear if HIV-associated renal disease also played a role in the higher concentration of sCysC.

We did not observe a significant correlation between sCysC and sCr in the three groups. This might have been due to non-renal factors influencing sCr and/or sCysC, such as FIV or hyperthyroidism, hydration status and drugs.[54] Sex and age distributions differed between the groups. In particular, male neutered cats were overrepresented in the FIV-infected group, which is similar to other reports.[55-57] However, no effect of age and sex on sCysC could be observed in a recent study by our group. The effect of age and gender on uCysC has not yet been evaluated.[14] Besides non-renal factors, other reasons might explain the lack of correlation between sCr and sCysC. Serum CysC could be influenced by early mild kidney dysfunction or might not be a reliable GFR marker. Several healthy cats, cats with hyperthyroidism and FIV-infected cats had USG < 1.035 without concurrent azotaemia. It is hypothesized that normal range for feline USG is between 1.001 and 1.065[58] or even 1.080,[59] but no large-scale studies have confirmed this. Because GFR was unavailable in the majority of the included cats, we cannot rule out that none of our included cats had early CKD, which might have affected the results of the present study. The GFR of healthy cats and cats with CKD measured with a gold standard method needs to be correlated with both sCr and sCysC, to study the sensitivity of sCysC to detect early kidney dysfunction in cats.

Our study is limited by the absence of a gold standard method to measure GFR. We cannot exclude that early kidney impairment was absent in our studied groups. A second limitation was the use of NSAIDs and antibiotics in the cats with FIV prior to inclusion, which might have influenced kidney function.

Conclusion

Cats with hyperthyroidism had a significantly higher sCysC concentration and most hyperthyroid cats had higher uCysC/uCr compared with healthy cats. However, based on the RI of sCysC, hyperthyroid cats cannot be distinguished from the healthy cats. Our study suggests that sCysC might not be a reliable GFR marker in hyperthyroid cats. Further studies with GFR measurement are required to reveal the mechanism of a possible increased sCysC in feline hyperthyroidism. Further studies must reveal the mechanism of the increased uCysC/uCr ratio in hyperthyroid cats.

Feline immunodeficiency virus infection did not influence sCysC, in contrast to HIV. Only a few cats had uCysC/uCr above the detection limit. Further studies are required to show if only a subset of FIV cats have tubular dysfunction or that only severe tubular dysfunction is detected by measuring uCysC.

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End Notes

[a] Witness® FeLV-FIV, Synbiotics, Lyon, France

[b] Advia 2120, Siemens, Brussels, Belgium

[c] Architect C16000, Abbott Max-Planck-Ring, Wiesbaden, Germany

[d] Immulite 2000 system, Siemens, Brussels, Belgium

[e] IQ 200 SPRINT, microscopic system, Instrumentation Laboratory, Zaventem, Belgium

[f] Iricell velocity, chemical system, Instrumentation Laboratory, Zaventem, Belgium

[g] Wask Copan, MLS, Vitek 2 system, BioMerieux, Brussels, Belgium

[h] BN Prospec nephelometric immunoassay, Siemens, Marburg, Germany

[i] SAS version 9.3, SAS Institute Inc, Illinois, USA

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SECTION §5.2

EVALUATION OF CYSTATIN C AS MARKER FOR THE DETECTION OF CHRONIC KIDNEY DISEASE IN CATS

Liesbeth F.E. Ghys[1], Dominique Paepe[1], Hervé P. Lefebvre[2], Brice S. Reynolds[2], Siska Croubels[3], Evelyne Meyer[3], Joris R. Delanghe[4], Sylvie Daminet[1]

[1] Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium

[2] Unité de Recherche Clinique, École Nationale Vétérinaire de Toulouse, Université de Toulouse, Chemin des Capelles 23, Toulouse Cedex 3, France

[3] Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820, Merelbeke, Belgium

[4] Department of Clinical Chemistry, Microbiology and Immunology, Faculty of Health Medicine and Life Sciences, Ghent University, De Pintelaan 184, B-9000, Ghent, Belgium

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Summary

Serum cystatin C (sCysC) and urinary cystatin C (uCysC) are potential biomarkers for early detection of feline chronic kidney disease (CKD). An in-depth clinical validation is required.

We aimed to evaluate CysC as marker for feline CKD. We compared sCysC and uCysC between CKD and healthy cats, correlated sCysC and sCr with glomerular filtration rate (GFR) and calculated sensitivity and specificity of sCysC for detecting decreased GFR. We also aimed to compare assay performance of the turbidimetric assay (PETIA) with the previously validated nephelometric assay (PENIA).

Ninety cats were included: 49 CKD and 41 healthy cats. Serum CysC and uCysC were measured in all cats. Based on plasma exo-iohexol clearance test (PexICT), sCysC was evaluated to distinguish normal, borderline and low GFR. Sensitivity and specificity to detect PexICT < 1.7 mL/min/kg were calculated. PETIA was validated and sCysC results of PENIA and PETIA were correlated with GFR. Statistical analysis was performed using general linear modelling.

Serum CysC and uCysC were significantly higher ($P < 0.001$) in CKD cats. Urinary CysC was detected only in 35/49 CKD cats. R^2 values between GFR and sCysC or sCr were 0.39 and 0.71 respectively. Sensitivity and specificity were 22% and 100% for sCysC and 83% and 93% for sCr. Serum CysC could not distinguish healthy from CKD cats, nor normal from borderline or low GFR, in contrast to sCr.

Serum CysC is not a reliable GFR marker in cats and uCysC could not be detected in all CKD cats.

Keywords: Feline; Chronic kidney disease; Glomerular filtration rate; Early diagnosis; Validation; Creatinine

Introduction

Chronic kidney disease (CKD) is common in geriatric cats, with a prevalence from 30% up to 40% in cats older than 10 years.[1-3] Since CKD is an irreversible and progressive disease, early detection and treatment is of major importance, aiming to slow down disease progression and to improve quality of life and longevity.[4,5] Glomerular filtration rate (GFR) is considered the gold standard variable to evaluate kidney function, but measurement is time-consuming and unpleasant for the patient. Therefore, the indirect GFR markers, serum creatinine (sCr) and urea, are routinely measured to estimate GFR. However, these markers are insensitive, since their serum concentration only increases when approximately 75% of the functional renal mass is lost.[6] Moreover, they are both influenced by muscle mass, age, feeding status, sex and intra-individual variation.[7-9] All those disadvantages support the need for new indirect biomarkers that can be measured easily and reliably.

Cystatin C (CysC), a 13 kDa protein, is a proteinase inhibitor, produced in every nucleated cell at a constant rate, that is responsible for intracellular protein catabolism.[10,11] Most of the properties required for an ideal endogenous GFR marker apply for CysC.[12] Compared to sCr, several human[13-16] and canine studies[17,18] have shown a better correlation of sCysC with GFR. In addition, urinary Cystatin C (uCysC) is a biomarker for tubular damage in humans[19,20] and dogs.[21] In a pilot study, our group observed a significant difference in sCysC and uCysC concentration between CKD and healthy cats. We also validated the human particle enhanced nephelometric immunoassay (PENIA) for feline CysC measurement[22] and established a reference interval (RI) of 0.58–1.95 mg/L for sCysC. In addition, we demonstrated that there is no influence of breed, age and sex on feline sCysC[7] and that it is not mandatory to fasten cats prior to evaluation of feline sCysC.[23] These findings make sCysC a promising marker to estimate GFR in feline medicine.

Three human CysC quantitation devices are currently available: ELISA,[24] particle enhanced turbidimetric assay[14] (PETIA) and particle enhanced nephelometric assay (PENIA).[25] The latter two analytical methods are more suitable for clinical use. No commercial veterinary assays are currently available, which requires validation of human assays. The assay validation of both PENIA and PETIA has been described in Chapter III.

The objectives of this study were fourfold. Firstly, sCysC and uCysC were compared between a large number of CKD and healthy cats. Secondly, the correlation of sCysC and sCr with GFR measured using plasma exogenous creatinine clearance test (PECCT), plasma endo-iohexol clearance test (PenICT) and plasma exo-iohexol clearance test (PexICT), was compared. In addition, the sensitivity and specificity of sCysC to detect decreased GFR were determined and compared with sCr. Thirdly, to determine which assay would be most suitable for clinical use, PENIA versus PETIA measurements of sCysC were each correlated with GFR estimated by PECCT, PenICT and PexICT.

Materials and methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Local Ethical and Deontological Committee of the Faculty of Veterinary Medicine, Ghent University (EC2011_197). All owners whose animal participated in the study signed an informed consent.

Study population

Adult CKD and healthy cats were included, regardless of breed and gender. In all cats, physical examination, complete blood cell count (CBC), serum biochemistry (BC) – including total thyroxine (TT4) measurement in cats older than 6 years – and urinalysis were performed to assess the general health status. Diagnosis of CKD was made prior to inclusion. Cats were diagnosed with CKD based on the presence of compatible clinical and laboratory findings (i.e. renal azotemia (sCr and urea exceeding the RI we established in Chapter IV §4.2, i.e. (64.5–161.8 µmol/L) for sCr and (6.2–12.7 mmol/L) for serum urea) and urine specific gravity (USG) < 1.035). Cats with CKD were classified into four stages according to the IRIS guidelines.[26] Cats with borderline sCr, but abnormal renal ultrasonographic findings at time of inclusion, with available follow-up data confirming CKD, were also included. CKD cats with evidence of significant concurrent systemic diseases based on their history, physical examination, CBC, BC or urinalysis were excluded. In CKD cats, treatment with angiotensin-converting enzyme (ACE)-inhibitors, calcium antagonists or angiotensin receptor blockers (ARB) had to be ceased at least one week prior to inclusion. Renal diet and phosphorus binders did not have to be withdrawn.

Cats with lack of significant abnormalities in history, physical examination, CBC, BC and urinalysis were defined as "healthy". Criteria for normal urinalysis were: USG > 1.035, inactive urine sediment, urinary protein:creatinine ratio (UPC) < 0.4 and negative bacterial urine culture.[27] Healthy cats receiving medication, within one month prior to inclusion, with potential effect on the kidney i.e. non-steroidal anti-inflammatory drugs (NSAIDs) corticosteroids, antibiotics, β-blocking agents, calcium antagonists, and ACE-inhibitors or ARB, were excluded.

Procedures

A standard physical examination was performed in all cats including systolic blood pressure (SBP) measurement using the Doppler ultrasonic technique and a standardized procedure according to the ACVIM consensus guidelines.[28] Cats were considered hypertensive if SBP > 160 mmHg.[28] Thyroid gland palpation was performed in cats older than 6 years, as previously described.[29] All cats were fasted at least 10 h before the sampling procedure. Five mL of blood was taken by jugular venipuncture using a 23 G needle. After centrifugation (5 min at 1931 x *g*), serum was analysed the same day. The CBC[a] and BC[b] were performed in all cats. Ten mL of urine was taken by cystocentesis with a 22 G needle. The USG was determined using a manual refractometer[c]. Urinalysis consisted of a urinary dipstick test,[d,e] measurement of UPC,[f] sediment analysis and bacterial culture.[g] Urine was centrifuged (3 min at 355 x *g*) and the urinary sediment was analysed within 30 min according to Paepe et al.[30] The supernatant and remaining serum were divided in aliquots of 300 µL and stored at -72 °C until batched analysis.

Glomerular filtration rate was measured by the combined plasma exogenous creatinine-iohexol clearance test (PEC-ICT), using a protocol previously described by van Hoek et al.[31] Briefly, a 22 G catheter was placed in the cephalic vein. Creatinine was dissolved in 4 mL of 0.9% sodium chloride. First iohexol[h] (64 mg/kg (0.1 mL/kg)), followed by Cr (40 mg/kg) and 3 mL of 0.9% sodium chloride was injected. Blood samples were taken by jugular venipuncture just before the injection and 5, 15, 30, 60, 120, 180, 360, 480, 600 minutes after injection. The samples were placed in EDTA tubes, centrifuged (5 min at 1931 x *g*) and plasma was stored in aliquots of 300 µL at -72 °C until analysis.

Assays

Serum CysC and uCysC were analysed with PENIA[k] using the nephelometer,[l] and with PETIA[i] using the Cobas auto-analyser.[j] Both assays were validated for feline CysC determination (Chapter III). Urinary CysC was expressed as a ratio to the urinary Cr (uCr) concentration, to compensate for differences in urine flow rates.[32] Plasma Cr was analysed with an enzymatic assay[q] and plasma concentrations of the stereo-isomers endo-and exo-iohexol were determined by high performance liquid chromatography with ultraviolet UV detection, both validated by van Hoek et al.[31] Serum Cr measured in BC[b] was analysed with a modified Jaffe assay, validated by a veterinary commercial laboratory.[n]

Pharmacokinetics for GFR determination

All analyses were performed using WinNonlin.[o] The data were subjected to non-compartmental analysis, as described by Watson et al.[33] The area under the plasma concentration versus time curve (AUC) was calculated using the trapezoidal rule with extrapolation to infinity. The plasma clearance of Cr, endo- and exo-iohexol was determined by dividing the dose administered by AUC and indexed to bodyweight (mL/min/kg).

Statistical analysis

Statistical analyses were performed using statistical software[p] and at the 0.05 significance level. The effect of status (CKD, healthy) on sCysC and uCysC, the effect of the IRIS stage on sCysC and uCysC, the relationship between uCysC/uCr and UPC and the comparison between sCysC measured with PENIA versus PETIA were tested by ANOVA. In case of a significant effect, pairwise comparisons were performed with the Tukey's test. If the uCysC concentration was < limit of detection (LOD), it was arbitrarly fixed to zero.

The correlation between GFR and either sCysC or sCr were determined with ANOVA using general linear model (GLM): Marker = μ + GFR + ε with marker being sCr, sCysCPENIA, sCysCPETIA, 1/sCr, 1/sCysCPENIA, 1/sCysCPETIA; GFR is GFR Cr, GFR exo, GFR endo; ε, error term of the model. For each marker, the R^2 of the regression was determined.

We also studied if sCysC could distinguish cats with normal, borderline or low GFR, based on PexICT, compared with sCr. The cut-off concentrations for normal GFR were

defined as GFR ≥ 1.7 mL/min/kg, for borderline GFR as GFR 1.2–1.7 mL/min/kg and for low GFR as GFR < 1.2 mL/min/kg, as determined previously by our group.[34]

Sensitivity and specificity of sCr and of sCysC to detect decreased GFR (PexICT < 1.7 mL/min/kg) were calculated. "Positive test" and "negative test" were defined as > respectively < 161.8 µmol/L for sCr, and > respectively < 1.95 mg/L for sCysC, based on the RIs we established in a previous study.[7] The nonparametric receiver-operating-characteristic (ROC) curve for sCysC and sCr were additionally configured.

Results

Study population

In total, 90 cats were recruited (age range: 1.1 years to 19 years), namely 49 CKD and 41 healthy cats.

For the 49 CKD cats, breed distribution was: one Siamese, one Oriental Shorthair, one Persian, one Maine Coon, one Burmese, two Ragdolls, two Birmans, four British Shorthair cats and 36 DSH/DLH. Four cats were female intact, 19 female neutered and 26 male neutered. Mean ± SD age was 10 ± 4.7 years and mean ± SD body weight 4.1 ± 1.2 kg. One cat had IRIS stage 1 non proteinuric CKD and was diagnosed based on ultrasonographic findings, low USG and borderline sCr. The cat had IRIS stage 2 CKD and IRIS stage 3 CKD, five months and two years after inclusion, respectively. Twenty cats had IRIS stage 2 CKD, of which five were proteinuric (UPC $\geq$ 0.4), five borderline proteinuric (UPC (0.2–0.4)) and 10 did not have proteinuria. Thirteen cats had IRIS stage 3 CKD. Six of those cats were proteinuric, three were borderline proteinuric and the other four cats did not have proteinuria. Fifteen cats had IRIS stage 4 CKD, of which 11 were proteinuric and four borderline proteinuric. One cat with severe proteinuria (UPC = 4.22) also had glucosuria without hyperglycemia. Three CKD cats were treated with ACE-inhibitors but therapy was ceased one month prior to inclusion. One CKD cat was treated with ARB, and therapy was stopped two weeks prior to inclusion.

For the 41 healthy cats, breed distribution was: one Birman cat, one Persian cat, two Ragdolls, two British Shorthair and 35 domestic short-or longhair (DSH/DLH) cats. Two cats were female intact, 25 female neutered and 14 male neutered. Mean ± SD age was 9.9 ± 3.5 years and mean ± SD body weight 4.4 ± 1.2 kg.

Systolic blood pressure measurement was performed in 45 CKD and 37 healthy cats. The other eight cats were not cooperative enough, to reliably determine SBP. Four CKD cats were hypertensive, of which only one was proteinuric. Also four of the healthy cats had SBP > 160 mmHg. Those cats were very stressed during the measurement, so white-coat hypertension is a likely explanation.[28] Systolic blood pressure, sCr, serum urea, sCysC, USG, UPC, of both CKD and healthy cats are presented in Table 1.

Table 1. Descriptive statistics of both CKD and healthy cats. The variables are presented as mean ± SD. The level of significance is indicated by [a] ($P < 0.01$) and [b] ($P < 0.001$).

Variable (unit)	**CKD (*n* = 49)**	**Healthy (*n* = 41)**
SBP (mmHg)	135 ± 27	142 ± 20
sCr (µmol/L) [b]	358.7 ± 223.6	108.9 ± 23
Serum Urea (mmol/L) [b]	26.6 ± 19.4	9.4 ± 2.1
sCysC (mg/L) [b]	1.4 ± 0.5	1.0 ± 0.3
USG [b]	1.019 ± 0.009	1.045 ± 0.007
UPC [a]	0.67 ± 0.92	0.21 ± 0.14
uCysC/uCr (mg/mol) [b]	291 ± 411	0.32 ± 0.97

CKD, chronic kidney disease; SBP, systolic blood pressure; sCr, serum creatinine; sCysC, serum cystatin C; USG, urine specific gravity; UPC, urinary protein:creatinine ratio; uCysC/uCr, urinary cystatin C:creatinine ratio.

Comparison of sCysC and uCysC between cats with CKD and healthy cats

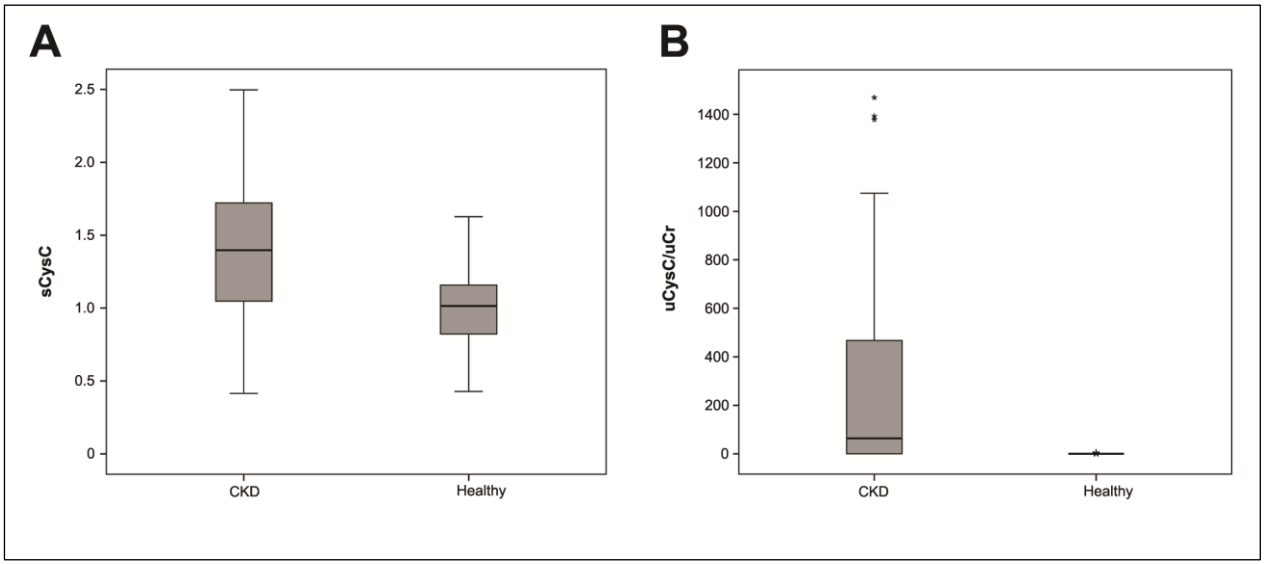
Serum CysC was measured in all cats and uCysC in 44 CKD cats and all healthy cats. Urinary CysC was < LOD (0.049 mg/L) in 15/44 CKD cats and in all but five healthy cats. We observed that there was a significant effect ($P < 0.001$) of the status (CKD or healthy) on sCysC and uCysC/uCr with significantly higher concentrations in CKD cats (Table 1). The corresponding boxplots of sCysC and uCysC/uCr of healthy cats and CKD cats are presented in Figure 1, illustrating the overlap in sCysC between the groups. The IRIS stage also had a significant positive effect ($P < 0.001$) on both sCysC and uCysC/uCr, with increasing mean concentrations as IRIS stage increased (Table 2). However, R^2 between IRIS stage and CysC was weak (0.31 for sCysC and 0.29 for uCysC). Also UPC had a significant effect ($P < 0.001$) on uCysC/uCr in the whole population and in cats with CKD. R^2 was 0.54 for the whole population and 0.50 for the cats with CKD.

Table 2. Serum CysC (sCysC) and urinary cystatin C:creatinine ratio (uCysC/uCr) measured met the nephelometric assay in the healthy cats and cats with CKD for each IRIS stage. Data are presented as mean ± SD. *n* represents the number of cats.

Status	sCysC (mg/L)	uCysC/uCr (mg/mol)
Healthy (*n* = 41)	1.0 ± 0.28	0.32 ± 0.95
CKD IRIS stage 1 (*n* = 1)	1.065	7.40
CKD IRIS stage 2 (*n* = 20)	1.26 ± 0.40	123.62 ± 374.4
CKD IRIS stage 3 (*n* = 13)	1.50 ± 0.50	254.0 ± 206.0
CKD IRIS stage 4 (*n* = 15)	1.67 ± 0.57	526.80 ± 489.87

CKD, chronic kidney disease; IRIS, international renal interest society; SD, standard deviation; LOD, limit of detection.

Figure 1. Boxplot of serum cystatin C (sCysC) (mg/L) **(A)** and the urinary cystatin C: creatinine ratio (uCysC/uCr) (mg/mol) **(B)** for 49 cats with chronic kidney disease (CKD) and 41 healthy cats.



Comparison of correlation between GFR and sCysC versus sCr

The PEC-ICT was performed in 17 CKD and 15 healthy cats. The mean ± SD Cr, endo- and exo-iohexol clearances of the CKD and healthy cats are presented in Table 3. In one healthy cat, the serum sample 60 min after injection was not available, and therefore the GFR of that cat was calculated based on nine samples instead of 10 samples. Both for the PENIA and the PETIA, there was a significant correlation between GFR and sCysC. The scatter plots of sCr and sCysC PENIA, sCysC PETIA versus PexICT are presented in Figure 2. The other GFR-markers showed comparable results. The regression coefficients with *P*-values are presented in Table 4.

Table 3. Mean ± SD of plasma clearance (mL/min/kg) of creatinine, exo-iohexol and endo-iohexol in CKD and healthy cats.

	CKD (*n* = 17)	**Healthy (*n* = 15)**
PECCT	0.9 ± 0.3	2.3 ± 0.6
PexICT	0.9 ± 0.4	2.1 ± 0.5
PenICT	1.2 ± 0.5	2.9 ± 0.7

Cr, creatinine; CKD, chronic kidney disease; PECCT, plasma exogenous creatinine clearance test; PexICT, plasma exo-iohexol clearance test; PenICT, plasma endo-iohexol clearance test.

Figure 2. Scatter plots of the glomerular filtration rate (GFR) determined with a plasma exogenous iohexol clearance test (PexICT), cystatin C analysed with the particle enhanced nephelometric immunoassay (sCysC PENIA) **(A)** and cystatin C analysed with the particle enhanced turbidimetric immunoassay (sCysC PETIA) **(B)** and serum creatinine (sCr) **(C)**.

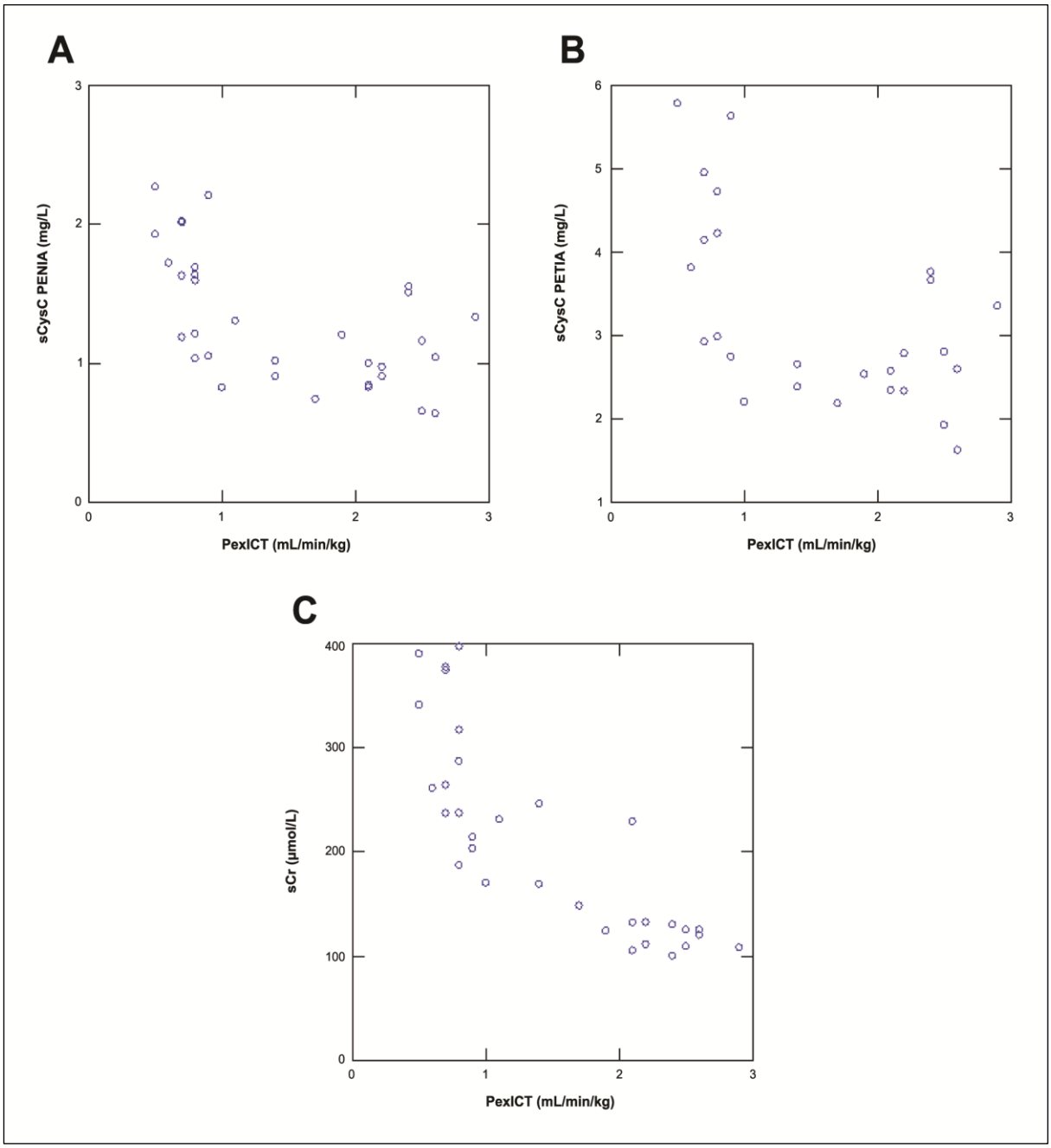


Table 4. R^2 values and associated *P*-values of sCr and sCysC measured with the PENIA and PETIA versus plasma clearance of creatinine, exo- and endo-iohexol.

	PECCT	**PexICT**	**PenICT**
sCysC PENIA	0.46 ($P < 0.001$)	0.34 ($P < 0.001$)	0.37 ($P < 0.001$)
sCysC PETIA	0.44 ($P < 0.001$)	0.31 ($P = 0.003$)	0.36 ($P = 0.001$)
sCr	0.74 ($P < 0.001$)	0.68 ($P < 0.001$)	0.67 ($P < 0.001$)

sCr, serum creatinine; sCysC, serum cystatin C; PENIA, particle enhanced nephelometric immunoassay; PETIA, particle enhanced nephelometric immunoassay; PECCT, plasma exogenous creatinine clearance test; PexICT, plasma exo-iohexol clearance test; PenICT, plasma endo-iohexol clearance test.

Determination of sensitivity and specificity of sCysC to detect decreased GFR

Results from the clearance test demonstrated that one cat classified as "healthy" actually had borderline GFR and another cat classified as "healthy" had low GFR. In addition, one "CKD" cat actually had borderline GFR and another had normal GFR. The boxplots of sCysC and sCr from cats classified with normal, borderline and low GFR are presented in Figure 3. For sCysC, the overlap was much larger compared to sCr between cats with normal GFR, cats with borderline and low GFR. Serum CysC exceeded the RI previously established by our group, in only four of 16 cats with low GFR. In contrast, sCr exceeded the RI in all of them. Indeed, the sensitivity of detecting decreased GFR (< 1.7 mL/min/kg) was 22% for sCysC compared with 83% for sCr. In contrast, the specificity for sCysC was 100% compared with 93% for sCr. The ROC is presented in Figure 4.

Figure 3. Boxplot of serum cystatin C (sCysC) (A) and sCr (B) for cats with normal GFR (GFR ≥ 1.7 mL/min/kg), borderline GFR (GFR (1.2–1.7 mL/min/kg)) and low GFR (GFR < 1.2 mL/min/kg). GFR determination was based on an exo-iohexol clearance test (PexICT).

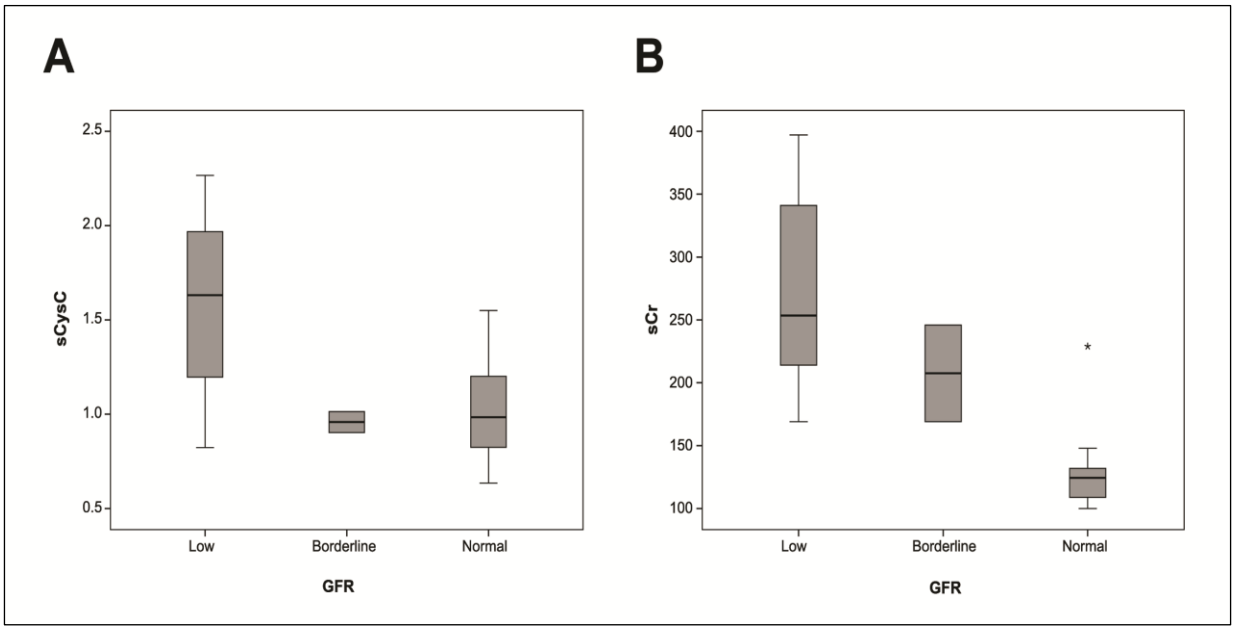
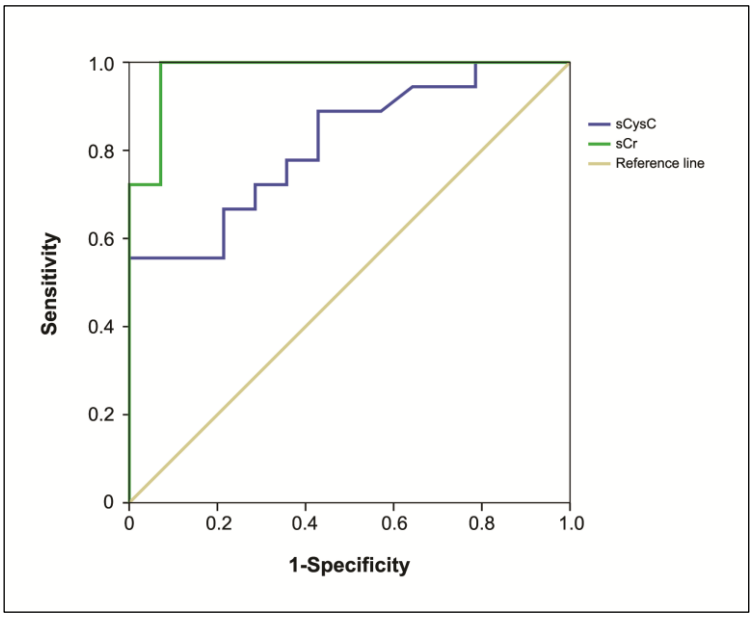


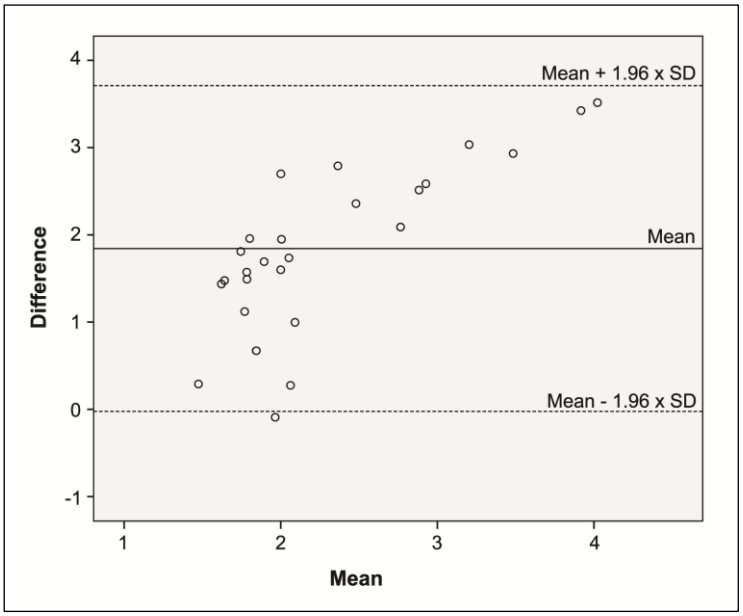
Figure 4. Nonparametric receiving-operating-characteristics (ROC) plots of the sensitivity and specificity of sCysC and sCr for distinguishing cats with normal and reduced GFR (< 1.7 mL/min/kg) determined with plasma exo-iohexol clearance test (PexICT).



Comparison between PETIA and PENIA for sCysC analysis

The two methods were highly correlated (R^2 = 0.94, P < 0.001), but sCysC concentrations measured with PETIA was significantly higher (P < 0.001) than those measured with PENIA. No significantly better correlation between sCysC PETIA and GFR (whatever the marker) than between sCysC PENIA and GFR could be observed (Fig 2, Table 4). Figure 5 presents the Bland-Altman Plot in which PENIA and PETIA are compared. The mean ± SD difference was calculated as 1.84 ± 0.95 mg/L. The limits of agreement are wide, and the difference tends to get larger as the mean increases.

Figure 5. Bland-Altman Plot of serum cystatin C analysed with the particle enhanced tubidimetric immunoassay (PETIA) and particle enhanced nephelometric immunoassay (PENIA).



Discussion

The striking results of this study are: i) sCysC was significantly higher in cats with CKD, compared with healthy cats, but a significant overlap was present; ii) urinary CysC was not present in all CKD cats; iii) the correlation between GFR measured with PEC-ICT and sCysC was weaker than that between GFR and sCr, regardless of the sCysC assay.

Although sCysC was significantly higher in CKD versus healthy cats, several other findings disfavor feline sCysC: an obvious overlap in sCysC between both groups was present, as previously described in cats[22,35] and dogs.[36] In addition, only six of the 49 CKD cats had sCysC above the upper limit (1.95 mg/L) of the RI, determined in a previous study.[7] Further, although significant, the correlations between sCysC and IRIS stage were weak and probably not clinically relevant, since not all cats with CKD IRIS stage 3 or 4 had a higher sCysC concentration compared with the healthy cats or cats with CKD IRIS stage 2.

Urinary CysC was also significantly different between CKD and healthy cats. In contrast with sCysC, an overlap for uCysC was absent. As for sCysC, a significant but weak correlation between uCysC/uCr and the IRIS stage was present, and not all CKD cats with IRIS stage 3 or 4 had higher uCysC/uCr compared to healthy cats and CKD cats with IRIS stage 2. Indeed, five of the 41 healthy cats had a detectable uCysC concentration. With normal kidney function CysC is completely reabsorbed and catabolized in the tubules.[37,38] Small quantities can still be found in the urine, but one would expect this concentration to be < LOD.[39] Two of the five healthy cats with detectable uCysC were borderline proteinuric. None of those five cats had overt proteinuria, azotemia or isosthenuric urine, so CKD is unlikely. Nevertheless, no follow-up or GFR results are available for those cats, so early CKD cannot be excluded. Unexpectedly, 15 of the 49 CKD cats had uCysC < LOD, of which two cats were proteinuric and two borderline proteinuric. In a previous study[40] of our group, uCysC could also not be detected in five of the 10 included cats with CKD. Those observations are surprising. Most of the cats with CKD have tubulo-interstitial lesions. Therefore, we expected detectable uCysC in most of the CKD cats. However, without histopathology, a more atypical form of CKD cannot be excluded. In contrast, uCysC seemed to be valuable as marker for local proximal damage in hyperthyroid cats.[41] Therefore, the

prognostic value and ability to detect early tubular damage of uCysC should be evaluated further with investigation of renal biopsies.

The major objective of the present study was to compare the correlation of GFR with sCysC and sCr. In contrast with human studies[14,15,42] and studies in dogs[17,18] a weaker correlation was found between PECCT, PenICT, PexICT and sCysC measured with PENIA or PETIA compared with sCr. In addition, the use of the inverse values increased R^2 value for sCr, but not for sCysC PETIA or sCysC PENIA. From these findings we can conclude that sCysC does not appear to be advantageous over sCr for detection of feline CKD.

Our results are different from a similar study in cats, demonstrating a significantly better correlation of sCysC with PICT compared to that with sCr.[35] In the study of Poświotowska-Kaszczyszyn a one-compartmental model and the slope-intercept method corrected with the Brochner-Mortensen formula was used to calculate GFR.[43] It has been shown that one-compartmental models overestimate true GFR, due to underestimation of AUC, and the slope-intercept method with the Brochner-Mortensen formula can cause increasing errors with increasing clearances.[44] Since we used a different method for GFR calculation, it is difficult to compare the results from the present study with the study from Poświotowska-Kaszczyszyn. In addition, we determined GFR with three different markers (Cr, endo-and exo-iohexol) to evaluate sCysC. However, no better correlation of each of the three methods with sCysC could be observed.

Early kidney impairment in some of the healthy cats could not be excluded. Therefore, in the subgroups in which GFR was measured, we correlated sCysC and sCr with renal function. Only two of the 15 cats previously classified as “healthy” had low and borderline GFR respectively. These two cats did not show high sCysC value exceeding the RI. Serum CysC overlapped between cats with low, borderline and normal GFR, indicating that sCysC cannot distinguish between those three groups. For sCr, the overlap was less severe and could mainly be observed between cats with low and borderline GFR. In dogs on the other hand, a significantly better correlation between sCysC and PECCT[18] or PICT[17] has been shown. However, an overlap in sCysC between healthy dogs and dogs with CKD was also present,[17,45,46] but GFR measurement was only performed in one of those studies.[17] In contrast to canine studies,[17,18] no higher sensitivity of sCysC than of sCr to detect decreased GFR

could be observed. In contrast, a higher specificity was present, which means that if feline sCysC is elevated, CKD is definitely present.

The findings of the present study do not encourage the clinical use of feline CysC. However, suboptimal feline CysC determination cannot be completely ruled out. We obtained a signal with the PETIA and PENIA, but we cannot exclude that the human assays measure feline CysC suboptimal. Western blot analysis with antibodies from the PENIA[22] could not demonstrate good cross reactivity at 13 kDa, in contrast with the antibodies from the PETIA (Chapter III). For both assays, bands at 26 and 52 kDa were visible. In humans, it has been shown that denaturing agents or high temperature can cause di-or polymerization of CysC.[47] This is unknown for dogs and cats, but it could explain the higher molecular weight bands. Alternatively, the human polyclonal anti-human CysC antibodies might detect polymers and not 13 kDa feline CysC. Another explanation for suboptimal testing could be the relatively limited homology between human and feline CysC. A homology of 70% between feline and human CysC has been described,[48,49] but the epitope sequence to which the antibody binds is not provided by the manufacturers, which makes evaluation of cross reactivity between the anti-human CysC antibodies and feline CysC difficult. Therefore, a feline assay should be developed, followed by a re-evaluation of this marker. Until then, we do not recommend the use of feline CysC as renal marker in cats.

Conclusion

Serum CysC was not able to distinguish healthy cats from cats with CKD. Furthermore, uCysC was not present in all CKD cats, but studies with renal biopsies are needed to confirm the usefulness of uCysC as proximal tubular marker. Whatever the marker for GFR determination and assay for CysC measurement, a markedly weaker correlation between GFR and sCysC compared with sCr could be demonstrated. Therefore, we do not advise to use feline sCysC as an indirect marker for GFR.

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End Notes

[a] Advia 2120, Siemens, Brussels, Belgium

[b] Architect C16000, Abbott Max-Planck-Ring, Wiesbaden, Germany

[c] Atago, Tokyo, Japan

[d] Iricell velocity, chemical system, Instrumentation Laboratory, Zaventem, Belgium

[e] IQ 200 SPRINT, Instrumentation Laboratory, Zaventem, Belgium

[f] Immulite 2000 system, Siemens Healthcare Diagnostics, Marburg, Germany

[g] Wask Copan, MLS, Vitek 2 system, Biomerieux, Brussels, Belgium

[h] Omnipaque 300, GE Healthcare, Amersham Health, Wemmel, Belgium

[i] particle enhanced turbidimetric assay, Dako, Glostrup, Denmark

[j] Cobas C system, Roche Diagnostics Gmbh, Mannheim, Germany

[k] particle enhanced nephelometric assay, Siemens Healthcare Diagnostics, Marburg, Germany

[l] BN Prospec Nephelometer, Siemens Healthcare Diagnostics, Marburg, Germany

[m] Vettest, Idexx laboratories Europe B.V., Amsterdam, the Netherlands

[n] Med Vet Lab, Antwerp, Belgium

[o] WinNonlin version 4.0.1., Scientific Consulting Inc. Apex, NC, USA)

[p] Systat 12, Systat Software Inc

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CHAPTER VI

GENERAL DISCUSSION

Chronic kidney disease (CKD) is common in cats, with a prevalence ranging from 30%–40% in aged cats.[1-3] CKD is routinely diagnosed based on clinical findings, serum creatinine (sCr), urea and urine specific gravity (USG). However, these parameters have their limitations for the detection of early CKD, as described in part one of **Chapter I**. The limitations of the available tests encourage the need for a new renal marker, that can easily and reliably be measured.

Cystatin C (CysC) appears to have all characteristics required to be an ideal renal marker. At the start of this thesis, several studies in human medicine had described the advantages of serum Cystatin C (sCysC) over sCr: it has a better correlation with GFR,[4-12] is less influenced by muscle mass,[13,14] food intake,[15] and is a very stable protein that can be stored for more than 10 years at -25°C.[16] In addition, urinary CysC (uCysC) can also be used as a marker for tubular dysfunction. Indeed, it is normally completely catabolized in the proximal tubule and therefore CysC appearance in urine is an indicator of proximal tubular damage.[17,18] Due to all those advantages, interest raised to investigate this marker in veterinary medicine. In dogs, several studies claimed CysC to be a better glomerular filtration rate (GFR) marker than sCr.[19-22] In cats, contradictory results were available.[23,24] An overview of current knowledge on CysC in small animal medicine has been described in part two of **Chapter I**.

Before a new marker can be routinely used, it needs to be validated **(Chapter I)**. Validation is a term that is mostly used unfittingly. The correct definition is the evaluation of the quality of a measurement, in order to identify the presence of an analyte, and to evaluate the status of the test subject.[25] Prior to this thesis, several veterinary laboratories offered analysis of CysC. However, this marker was not yet appropriately validated. Moreover, in feline medicine, only few CysC studies had been performed.

As described in **Chapter II**, the objectives of this thesis were to evaluate

- how feline CysC could be appropriately measured
- the influence of biological factors on feline sCysC
- the influence of clinical factors on feline sCysC and uCysC
- if CysC was a better marker than sCr for the detection of feline CKD

1. Measurement of feline cystatin C

In **Chapter III**, analytical validation of feline CysC was performed. We determined how feline CysC can be measured. As no veterinary assays were available, we needed to evaluate if the available human assays were able to determine feline CysC. At the start of the study, three human assays were available: particle-enhanced turbidimetric immunoassay (PETIA), particle-enhanced nephelometric immunoassay (PENIA) and enzyme-linked immunosorbent assay (ELISA). Since the objective of our thesis was to evaluate if sCysC could be used in practice, we did not evaluate ELISA, as this test is labour-intensive, expensive and not suitable for daily use in practice. A disadvantage of using human assays for feline samples is that the obtained concentrations are relative values. We do not know the exact feline CysC concentration. Therefore, we wanted to produce purified feline CysC, to know which correction factor is required to calculate the actual measured feline CysC concentration. It was described that feline recombinant CysC can be expressed in *Escherichia coli* with antibody production.[26] Based on this information, we cloned the feline CysC gene into a plasmid, followed by cloning into an expression vector *(Escherichia coli)* with a fusion protein for protein purification. However, despite several attempts to produce recombinant feline CysC not only in *Escherichia coli*, but also in *Pichia pastoris* and mammalian cells, recombinant feline CysC could not be produced. Possible explanation is that the fusion protein could not be removed due to non-recognition, or that transcription of translation was stopped too early. Further experiments to produce feline CysC were not undertaken, since this was beyond the scope of this thesis. Therefore, the human CysC standard was used for the validation of CysC and relative feline CysC concentrations were measured.

Like ELISA, PENIA and PETIA are two assays based on antigen-antibody reaction. Therefore, cross-reactivity between the polyclonal anti-human CysC antibodies, used in the immunoassays, and feline CysC was demonstrated with Western blotting. Both healthy cats and CKD cats with and without proteinuria were selected. On Western blot with antibodies from PETIA, small bands could be demonstrated in urine and in serum of a non proteinuric cat with CKD. Western blot with antibodies from PENIA could demonstrate bands in urine samples from the proteinuric CKD cats, but no bands could be detected at the expected 13 kDa MM of either cat. Surprisingly, both for PENIA and PETIA, bands could be detected at 26 and 52 kDa, especially in serum. Only one study using PETIA antibodies has shown

photographs of Western blots with comparable findings, but these results were not discussed.[19] It has been described in humans that denaturing agents and high temperature conditions, result in polymer formation of CysC.[27] In our protocol, samples were boiled for 10 min at 95 °C and incubated with laemli buffer, which contains disulphide bond reducing agents, sodium dodecyl sulphate and dithiothreitol. In the study of Almy et al.[19] with comparable results, another reducing agent, β-mercapto-ethanol, prior to the electrophoresis step was added. Therefore, we assume that the bands at 26 kDa and 52 kDa, a two- and fourfold of 13 kDa respectively, were caused by polymer formation induced by the applied conditions. However, this could not explain the stronger signal in urine compared to serum. In humans, it has been shown that uCysC lacks 4 to 8 amino-acids at the N-terminal compared to the sCysC homologue, and that it remains reactive to anti-CysC antibodies from the PETIA.[17] However, it is unknown if this also applies for PENIA antibodies, and for feline CysC. Furthermore, both PENIA and PETIA antibodies are polyclonal rabbit anti-human CysC antibodies, but it is unlikely that the antibodies from PENIA and PETIA are identical. In addition, a homology of only 70% between feline and human CysC has been reported,[26,28] and the epitope sequence to which the human antibodies bind is not provided by the manufacturer. Therefore, a further evaluation of difference in cross reactivity between antibodies from PENIA and PETIA on one hand, and feline sCysC and uCysC on the other, is currently not possible. In addition, there is no information available on Western blot results with human samples. We could not exclude that CysC is not suited for Western blot analyses.

Although cross reactivity between feline CysC and the human CysC antibodies could not be clearly demonstrated, we analyzed a few feline samples with PENIA and PETIA and a signal could be detected. In a study in dogs, the PETIA antibodies gave similar results on western blotting[19] and sCysC could be detected in those dogs. Therefore, we assumed that feline sCysC could be detected with PENIA and PETIA. One study in dogs has compared PENIA with PETIA for measuring sCysC among dogs with different IRIS stages.[29] Clinically important differences in canine sCysC concentrations between all IRIS stages were observed for sCysC measured with PENIA, in contrast to sCysC measured with PETIA.[29] In addition, studies with human serum samples showed less interference of triglycerides, bilirubin, hemoglobin of PENIA compared with PETIA.[6,7,30] Based on the validation parameters, but also due to practical reasons, we analysed feline CysC with the human PENIA. In the pilot study described in **Chapter III**, a significantly higher sCysC concentration was observed in CKD cats compared to healthy cats, but an overlap was present. These preliminary findings

were confirmed by the study described in **Chapter V, §5.2.** More CKD cats were included in that study, and an overlap in sCysC between the different IRIS stages could be observed. In the pilot study of **Chapter III**, uCysC was measured in one cat with lower UTI, and this cat did not have higher uCysC/uCr compared to the other cats. This was a preliminary finding, since we did not evaluate the effect of hemoglobin on measurement of uCysC in more samples or with PETIA, so we do not know if hemoglobin might have influenced our measurement. It is also worth mentioning that PENIA can be used with only one specialized automated nephelometer in contrast to PETIA, causing less analytical variability. However, to decide which assay was most suited for use in practice, a thorough analytical validation of both assays **(Chapter III)** was needed and correlation of GFR with feline sCysC measured with both assays **(Chapter V, §5.2)** practice, was performed.

In **Chapter III**, analytical validation of both PENIA and PETIA was performed according to the recommendations described in **Chapter I**. PENIA showed good precision and linearity, both for serum and urine. In contrast, sCysC and uCysC appeared mildly less linear for PETIA. In addition, PETIA had a high detection limit of 0.39 mg/L compared with 0.049 mg/L for PENIA, impeding CysC measurement in urine. In **Chapter V, §5.2**, sCysC was measured with PETIA and PENIA in both healthy and CKD cats and correlated with plasma exo-iohexol clearance test (PexICT). We observed significantly higher sCysC concentrations determined with PETIA compared to PENIA, but no significantly better correlation of PexICT and sCysC PETIA than of PexICT and sCysC PENIA. The Bland-Altman plot showed a mean difference higher than 0, indicating that PENIA and PETIA are systematically producing different results. For sCysC measurement, both PENIA and PETIA can be used, but for uCysC, we recommend PENIA. In addition, the findings from **Chapter V, §5.2** indicate that the same assay should be used, and sCysC results from PENIA and PETIA cannot be used interchangeably.

To use CysC in practice, it also mandatory to know at which temperature and for how long samples can be stored, before CysC concentration decreases. For the storage study **(Chapter IV, §4.1)**, only 4 CKD and 3 healthy cats were included, due to practical reasons. Because uCysC was undetectable in most included cats, only sCysC was investigated for this study. Based on our findings, samples can be stored at room temperature (RT) if sCysC is analyzed within 24 h and at 4 °C for 1 week and one freeze-thaw cycle is allowed. If longer storage is required, we advise storage at -20 °C and -72 °C for up to 5 months. Longer storage

is not recommended, since a 21% decrease in sCysC concentration was observed. Some studies in dogs[31] and cats[23] had measured plasma CysC (pCysC) instead of sCysC. To use in practice, it was important to know what the difference was between sCysC and pCysC and this had not been investigated earlier. Plasma CysC concentrations were significantly lower than sCysC concentrations, indicating that always the same medium must be used in patient follow-up.

The studies from **Chapter III** and **Chapter IV, §4.1** provided us the required information for CysC measurement. Feline sCysC can be measured both with PENIA and PETIA, feline uCysC is preferably measured with PENIA, the feline CysC concentrations can only be interpreted relatively, and prolonged storage of feline sCysC samples is not recommended.

2. Biological factors influencing feline serum cystatin C

In **Chapter IV**, **§4.1,** we investigated if it was mandatory to fasten cats prior to sCysC evaluation. This could be an advantage over sCr. If a renal marker can be measured regardless of feeding status, false positive or negative results due to feeding can be excluded. In cats, only two studies have investigated the effect of feeding on sCr. One study observed a postprandial increase,[32] whilst the other observed a decrease.[33] In the study of **Chapter IV**, **§4.1**, we investigated the effect of feeding versus fasting, and diurnal variation on both sCysC and sCr in a cross-over study with 10 healthy laboratory cats. There was no significant difference in sCysC and sCr between fasted and fed cats. For sCysC, our results were different from a similar study in dogs.[31] In that study, a postprandial decrease followed by an increase in pCysC could be observed. The authors attributed the decrease in pCysC to increased GFR, caused by increased renal blood flow. However, these dogs were fed a commercial diet, and dietary effect on GFR has only been demonstrated after consumption of raw and cooked meat.[34,35] Our findings for sCr were also different from previous studies in cats,[32,33] in which the authors attributed a postprandial increase to a high creatine content of the food[32] and the postprandial decrease to increased GFR.[33] An increased GFR postprandially has been demonstrated in cats fed a high protein diet,[36] but not in cats fed a normal protein diet, as was given in our study and the study from Reynolds and coworkers.[33] Currently, it is not proven that feeding cats a normal protein diet causes increased GFR. We only observed significant differences for sCysC between the morning and the evening samples, but the differences were small and probably not clinically relevant. For sCr no differences were observed in contrast to another study.[33] We observed large inter-individual differences in sCysC and sCr concentrations during the day, which could explain the differences between our study and the study from Reynolds and colleagues.[33]

Based on our findings, it is not mandatory to fasten cats prior to sCr and sCysC evaluation and samples can be taken at any moment during the day.

Even though sCysC concentrations are relative values, it is necessary to know what sCysC concentration is normal for healthy cats. In **Chapter IV**, **§4.2**, we established a reference interval (RI) for sCysC according to the ASVCP guidelines.[37] One hundred and thirty cats of different breeds, ages (young, middle-aged, old) and sex were included to

characterize biological variation and to determine if separate RIs would be required. Systolic blood pressure (SBP), sCr, urea, USG and urinary protein:creatinine ratio (UPC) were also compared between the different groups. We could not observe an effect of age, breed and sex on feline sCysC, as also observed by another research group.[24] This was an advantage over sCr. For sCr, we observed a small but significant difference between the separate age groups. However, a clear overlap was present. Therefore, no separate RIs were determined. In addition, no influence of breed on sCr could be observed, in contrast with other studies.[38-40] However, we included only 56 purebred cats of seven different breeds. In the studies from Paltrinieri et al. and Reynolds et al. more cats per breed were included, making these studies more representative. They proposed a breed-specific sCr RI for Birman, Maine Coon, Chartreux, Abyssinian and Persian Cats.[39,40] However, it should be kept in mind that their results cannot be extrapolated to other laboratory conditions.[39] In a study performed in our laboratory, sCr concentrations did not differ between healthy cats and Ragdoll cats.[41] We are convinced that further investigation is warranted, with larger populations of healthy purebred cats, and additional studies should be undertaken to understand the underlying causes for the differences.

We also observed interesting differences between the groups for other renal parameters. In our study age and sex influenced serum urea, which is known to be more influenced by extra-renal factors.[42,43] We observed a significantly higher UPC in male intact cats compared to female intact and female neutered cats, but not compared to male neutered cats. It is assumed that male intact cats have higher UPC than male neutered cats due to higher urinary cauxin concentrations, regulator of a pheromone precursor.[44,45] However, additional studies are needed, as only 10 male intact cats were enrolled and UPC values overlapped between the two groups. The systolic blood pressure (SBP) was significantly higher in geriatric cats compared to the middle-aged and young cats. Some studies observed an effect of age on SBP in cats,[46,47] whilst others did not.[48,49] None of our geriatric cats had SBP over 180 mmHg, and only two had SBP > 160 mmHg. Possible explanations are age-specific changes in the cardiovascular system or co-existing diseases such as declining kidney function or hyperthyroidism. Longitudinal studies evaluating SBP, renal and thyroid function are needed, to determine if SBP is independently age-related or related to co-existing diseases.

Since no effect of age, sex and breed on feline sCysC could be demonstrated, sCysC results from the entire population could be included in the RI calculations. According to the ASVCP guidelines, a minimum of 120 cats should be included, and the RI should be determined with a nonparametric method with the 2.5th and 97.5th percentile serving as lower and upper reference limit, respectively.[37] The obtained RI was 0.58–1.95 mg/L. This does not correspond with a previously reported RI of 0.34–4.11 mg/L.[23] However, in that study, pCysC was measured with PETIA, not validated at the time of that study. In addition, no clinical data of the cats were described, so concurrent diseases cannot be excluded. Finally, the RI was calculated with less than the required 120 cats. Remarkably, sCysC concentrations of CKD cats from previous studies,[24,50] fell within our RI. In the Polish study, GFR measurement was performed and sCysC was also measured with PENIA, but an overlap in GFR between CKD and healthy cats was observed, which could explain why sCysC from CKD cats fell within our RI.

It is mandatory to determine laboratory-specific RI.[37] Many inadequate RI are used in veterinary medicine., often due to an inadequate reference population.[37] The RI for sCr (44.2–141.2 µmol/L), used in the study of **Chapter III**, is based on sCr concentrations of cats aged 6 months to 1 year. This is probably not suited to interpret sCr of aged cats.[47] In the study of **Chapter IV §4.2**, 7 cats of our population exceeded that RI for sCr. None of these cats showed clinical signs of CKD or had a USG < 1.035, making early CKD unlikely. This encouraged the need for determination of laboratory-specific RIs according to general guidelines.[37,51] We calculated a sCr RI of 64.5–161.8 µmol/L according to the ASVCP guidelines.[37] Not only the RI has to be taken into account before sCr evaluation, also the assay variation has to be considered.[37] Serum Cr was measured with a modified Jaffe method, and none of our cats exceeded the upper reference limit (177 µmol/L) of a published RI for sCr measured with the modified Jaffe method.[52] Compared to the original Jaffe method, the modified Jaffe method minimizes the influence of pseudo-Cr chromogens i.e. proteins, ketones and glucose, which can overestimate true Cr.[53,54] However, the enzymatic method deals more effectively with the interferences, but a small overestimation is still possible compared with the former golden standard method, high performance liquid chromatography (HPLC).[55,56] Nevertheless, the modified Jaffe method is still most commonly used in Belgium and is less expensive than the enzymatic method. As described in **Chapter I**, we have to be aware that in human medicine, a new golden standard method i.e. gas-chromatography-isotope dilution mass spectrophotometry (IDMS) and a Cr standard are used, possibly also

influencing sCr results in cats.[53,57] In **Chapter IV §4.2**, 25 cats had USG < 1.035, without elevated sCr, or signs of CKD. More than two years after the inclusion, 20 of them were contacted again, and none of them had developed CKD. In **Chapter V §5.1**, we observed not only in six out of 28 healthy cats, but also in five out of 30 FIV-positive and nine out of 26 hyperthyroid cats, USG < 1.035 without concurrent azotemia. No GFR measurement was available in both studies, hence we cannot exclude that early kidney dysfunction was present in some cats. This was a limitation of both studies. However, it has been shown that USG has a daily variation,[58] and it is hypothesized that normal feline USG range is between 1.001-1.065[59] or even 1.080.[60] A recent study demonstrated that there was an association with USG < 1.035 and age and dietary moisture content in healthy cats.[61] In our study, not only aged, but also young cats had USG < 1.035. This was also observed in another study of our group and by Rishniw and colleagues.[61,41] An underlying cause could be identified in 22 out of 121 adult cats by Rishniw, in contrast with the young cats.[61] These three studies[41,61,62] emphasized the need for more studies investigating the clinical importance of USG < 1.035 in cats.

Even though our results are preliminary, since only small subgroups of cats were included, our observations encourage the need for studies evaluating the effect of biological factors on several renal parameters in large cat subgroups. Not only the factors age, breed and sex are important to evaluate, the within- and between-subject components of biological variation are also important to be considered, to decide if subject-based or population-based RI have to be used. The analyte's index of individuality should be determined, based on intra-individual, inter-individual and analytical variation.[63,64] For variables with high individuality, subject-based RI are more appropriate than population based RI.[64] In practice, subject-based RI refer to reference change values, calculated per analyte, based on individual biological variation.[63] This biological variation also includes imprecision, accuracy and total error of the laboratory assays.[63] Therefore, reference change values are also laboratory-dependent. For both sCr and sCysC, it was shown in dogs that population-based RI should be used.[65] This means that sCysC has no advantage over sCr for follow-up of a patient, in contrast to humans, since human sCysC had a higher index of individuality compared to sCr.[63,64,66] In cats, it has been shown that subject-based RI are more appropriate for sCr.[63] We intended to determine the index of individuality for feline sCysC, but the study was not undertaken, since the results of the study described in **Chapter V §5.2** disfavored feline sCysC over sCr.

The studies of **Chapter IV** showed that feline sCysC is not influenced by age, sex and breed and that the concentration is uninfluenced by feeding status. We also determined a RI according to the ASVCP guidelines of 0.58–1.95 mg/L for sCysC and of 64.5–161.8 µmol/L for sCr.

3. Clinical factors influencing feline CysC

Our previous studies showed more advantages of CysC over sCr at analytical and biological level. However, the clinical performance of feline CysC still needed to be elucidated.

3.1 Serum cystatin C

In **Chapter V**, **§5.1,** the influence of two common diseases on feline CysC was investigated. As hyperthyroidism and human immunodeficiency virus (HIV) may cause increased sCysC concentrations in humans,[67-69] we investigated the influence of both hyperthyroidism and feline immunodeficiency virus (FIV) on feline CysC. We included 30 cats with FIV, 26 hyperthyroid and 28 healthy cats. Serum CysC and uCysC were measured in all cats in addition to the routinely measured renal variables sCr, USG, UPC and total thyroxine (TT4). The hyperthyroid cats had significantly higher sCysC and uCysC/uCr ratio and UPC compared to the healthy cats in contrast to sCr, which was significantly lower. Our observations were comparable with human studies.[67,70] We did not observe a significant correlation between TT4 and sCysC, in contrast with sCr. In addition, none of the hyperthyroid cats exceeded the RI (0.58-1.95 mg/L) we established in **Chapter IV**, **§4.2**, and sCysC also seemed to overlap between the healthy and hyperthyroid cats. Not only in healthy and CKD cats **(Chapter V**, **§5.2)** but also in hyperthyroid cats, we demonstrated that sCysC is not a reliable GFR marker. The study in hyperthyroid cats was limited by lack of GFR measurement, but preliminary data with GFR measurement,[71] demonstrated no change in sCysC before and after treatment of hyperthyroidism in cats, in contrast to GFR and sCr.

In contrast to human studies,[68,72] no significantly higher sCysC concentration could be observed in FIV-positive cats. It was suggested in humans that sCysC was associated with

HIV-replication,[73] but GFR was not measured by a gold standard method, so renal disease could not be excluded to play a role in the higher sCysC concentration.

In all three groups, we could not observe a correlation between sCr and sCysC. This could be due to non-renal factors, such as FIV, hyperthyroidism or hydration status, the influence of early/mild kidney dysfunction or absence of a correlation. The latter was confirmed in **Chapter V**, **§5.2.**

In the final study of this thesis, described in **Chapter V**, **§5.2,** we evaluated cystatin C as a marker for CKD by comparing sCysC and uCysC between CKD and healthy cats, by correlating sCysC and sCr with GFR and by calculating sensitivity and specificity for decreased GFR. Previously, we had observed an overlap in sCysC between CKD and healthy cats in the pilot study (**Chapter III).** In addition, 7 out of 10 CKD cats of our pilot study and 46/46 cats of the study of Poświatowska-Kaszczyszyn fell withing the RI (0.58-1.95 mg/L) we established in **Chapter IV, §4.2.** However, a limitation in our previous studies was the lack of GFR measurement, unmasking possible early CKD. Therefore, in the study of **Chapter V**, **§5.2,** GFR was measured with the combined plasma exogenous Cr-iohexol clearance test (PEC-ICT) in 17 CKD and 15 healthy cats. We observed no significantly better correlation between sCysC and GFR compared to sCr for none of the three methods (Cr, endo- and exo-iohexol). Our results were in contrast with the study of Poświatowska-Kaszczyszyn, in which they found a significantly better correlation of sCysC with PICT than of sCr.[24] The contradictory result of both studies can be explained by the methods of GFR estimation. Based on our findings, we conclude that there is no better correlation between GFR and sCysC than with sCr.

As in our pilot study, we observed a significantly higher ($P < 0.001$) sCysC concentration in 49 CKD cats compared with 41 healthy cats. We observed a significant ($P < 0.001$) effect of IRIS stage on sCysC, with increasing concentrations as IRIS stage increased. However, all observed correlations were poor and probably not clinically relevant. More importantly, we observed a significant overlap in sCysC between CKD and healthy cats, again, as in our pilot study in **Chapter III**. In the study of Poświatowska-Kaszczyszyn,[24] GFR was also found to overlap, explaining the overlap in sCysC between CKD and healthy cats. Therefore, in our study, we correlated sCysC and sCr with the degree of renal function i.e. correlation with GFR. This was based on plasma exo-iohexol clearance test (PexICT),

since it was shown that PexICT has the largest accuracy and reproducibility.[74] The cut-off concentrations for normal GFR were defined as GFR ≥ 1.7 mL/min/kg, for borderline GFR as GFR 1.2-1.7 mL/min/kg and for low GFR as GFR < 1.2 mL/min/kg, determined in a previous study of our group.[75] Only two cats defined as "healthy" had low and borderline GFR respectively. These two cats did not show high sCysC concentrations exceeding the RI (0.58-1.95 mg/L). Serum CysC overlapped between cats with low, borderline and normal GFR, indicating that sCysC cannot distinguish between those three groups. For sCr, the overlap was less severe, and mainly present between cats with low and borderline GFR. Other studies also showed that a mild decrease in GFR does not cause increased sCr.[59,76] In addition, the sensitivity of sCysC to detect decreased GFR (PexICT < 1.7 mL/min/kg) was only 22% compared with 83% for sCr, and the specificity 100% compared with 93% for sCr. This means that a cat with early CKD will not have increased sCysC prior to increased sCr. However, if sCysC is elevated, CKD is definitely present. Our findings are different from canine studies, in which a significantly better correlation between GFR and sCysC was observed compared with sCr.[20,22,77] However, an overlap in sCysC between healthy and CKD dogs could also be observed. Unfortunately GFR was measured in only two studies,[20.22] but no GFR data were provided in the study from Miyagawa et al. In the study from Wehner and colleagues, an overlap could be observed between dogs with borderline and normal GFR. Nevertheless, dogs with various diseases were included, which could have influenced sCysC concentration. Therefore, the reason for overlap of sCysC in dogs is currently unclear. Our study showed that sCysC is not a more sensitive marker for detection of feline chronic kidney disease than sCr.

3.2 Urinary cystatin C

Tubulo-interstitial nephritis is most commonly observed in cats with CKD,[78] making uCysC also a possible marker for detection of feline CKD. As observed in our pilot study in **Chapter III,** cats with CKD had significantly higher uCysC/uCr compared with healthy cats in the final study of **Chapter V**, **§5.2 ,** and no overlap was present. We observed a significant ($P < 0.001$) effect of IRIS stage on uCysC/uCr, with increasing concentrations as IRIS stage increased. Also UPC had a significant ($P < 0.001$) effect on uCysC/uCr. However, as for sCysC, the correlations were low and probably not clinically relevant. Nevertheless, some remarks have to be made. Five of the 41 healthy cats had detectable uCysC concentrations.

Since CysC is normally completely reabsorbed and catabolized in the kidney,[79,80] one would expect the concentration to be below the detection limit in the healthy cats. However, early CKD or tubular damage could not be excluded, since no GFR was performed and no kidney biopsies were taken in those cats, and no follow-up was available. In the CKD cats on the other hand, 15/49 had uCysC below LOD of which two were borderline proteinuric and two proteinuric. Since tubulo-interstitial lesions are most commonly present in cats with CKD, we expected presence of uCysC in all CKD cats. Both in the CKD and healthy cats, no other tubular markers were measured that could give an indication of presence or absence of tubular damage and no histopathology reports of the kidneys were available. We also observed presence of uCysC in hyperthyroid cats described in **Chapter V**, **§5.2.** Eighteen out of 26 hyperthyroid cats had a detectable uCysC concentration and a significant correlation with UPC was present. However, we observed a significant correlation between uCysC and sCysC in the hyperthyroid cats, indicating that uCysC is not independent from sCysC. For the FIV-positive cats on the other hand, only 4 out of 30 FIV-positive cats had detectable uCysC concentration. Three of those cats had received both antibiotics and NSAID's prior to sample collection. The effect of those drugs on CysC metabolism is unknown, but they may affect kidney function, which could have influenced our results.[81] In contrast to the hyperthyroid cats, not all proteinuric FIV-positive cats had detectable uCysC. Both glomerular and tubular pathologies have been described in FIV-positive cats.[82,83] As in the study described in **Chapter V**, **§5.1,** no biopsies were performed in the hyperthyroid and FIV-positive cats. Therefore, studies involving renal biopsy and correlation with other tubular markers are necessary to examine the ability of uCysC to detect early tubular damage.

4. Future perspectives

Our results showed that with the evaluated human assays, feline sCysC was not a reliable indirect marker to estimate GFR. Serum CysC was not able to distinguish healthy cats from cats with CKD. This was observed in a preliminary study in **Chapter III**, and was confirmed in **Chapter V**. Therefore, current data do not recommend to use feline sCysC as marker for feline CKD detection, at least with PENIA or PETIA.

Urinary CysC, a proximal tubular marker was not present in all CKD and hyperthyroid cats. However, we cannot conclude that uCysC is not useful. Indeed, tubulo-interstital lesions are most commonly observed in cats with CKD,[78] but both glomerular and tubular damage can be present in hyperthyroid cats and cats with CKD.[78,84] Additional studies with renal biopsy confirmation are valuable, to further investigate the use of uCysC in cats.

Are additional studies with feline sCysC valuable? It was a major disadvantage that no feline assays were available. Although, we obtained a signal with the human PENIA and PETIA, we could not be completely sure that feline CysC with MM of 13 kDa was measured appropriately. A homology of 70% between cat and human CysC has been described,[26,28] but epitope sequence to which the antibodies bind, has not been provided by the manufacturer. A feline assay should be developed, followed by a re-evaluation of feline CysC. Monoclonal antibodies to feline CysC have been produced,[26] but a commercial assay is not yet available. If new antibodies become available, it is worthwhile to investigate if these detect feline CysC. Until a feline assay is available, we do not recommend the use of feline CysC for kidney function evaluation.

This means that veterinary science needs to follow other pathways to detect early feline CKD. The use of limited sampling strategies for GFR estimation seems promising. Many different strategies have been used, and there is need for a consensus to define which method is most suitable, how many samples at which time points are required.[75,85-89] GFR in cats is mostly estimated by iohexol or exogeneous Cr. The analysis of iohexol still requires specialized devices, and analysis cannot yet be performed in-house. Exogenous Cr has the advantage that it can be analyzed more easily, but is not commercially available. Although the limited sampling strategy requires fewer samples, this method is still not practical for routine

use. In humans, formulas based on demographic variables to estimate GFR are routinely used. An attempt was made for cats, based on the routine renal variables sCr, urea and UPC, but the formulas have not been evaluated in a large cat population and specificity has not been investigated yet.[75]

Another possible renal marker is symmetric dimethylarginine (SDMA). It is an arginine residual and is released into circulation after proteolysis.[90] Plasma SDMA is increased in cats with CKD and correlates with plasma Cr concentration.[91] Two studies have found a similar correlation of SDMA and plasma Cr with GFR.[90,92] In addition, SDMA increased before sCr, which is a major advantage.[92] However, further studies are warranted. Firstly, only analysis of SDMA with the sophisticated HPLC-method has been described. Secondly, the marker was only tested with retrospective samples in a large group of laboratory cats,[92] and prospectively in a small number of client-owned cats.[90] Thirdly, GFR was estimated with the one-compartment method with only three blood samples, and might overestimate true GFR.[93] Fourthly, no RI according to the ASVCP guidelines or a cut-off concentration was determined.[37] Also the specificity needs to be further elucidated. Nevertheless, at this moment, this is the most promising marker for detection of feline CKD.

Besides early detection, we need to investigate which actions are required to slow down, arrest, or ideally, reverse disease progression.[94] We need to determine what therapies are of real value at the different stages of CKD and what therapies can improve quality and longevity of life.[94] We still have much to investigate in cats to understand CKD and prevent progression.

5. Conclusions of this thesis

In this thesis, we have investigated the possible use of CysC as marker for the detection of feline CKD. We have performed a thorough validation at analytical, biological and clinical level. First, we investigated how feline CysC can be measured in serum and urine and how samples can be stored. Since no feline assays were available, we validated the human nephelometric and turbimetric assay. Based on the validation results, we decided that sCysC can be analysed both with the turbidimetric and nephelometric assay, but that the human nephelometric assay was the best way to determine feline uCysC. Storage of feline sCysC for more than 5 months was not recommended. In a pilot study, we observed a significant difference in sCysC and uCysC between CKD and healthy cats, but an overlap between the two groups was present. At this point in the research, it was too preliminary to conclude that CysC did not have the required properties for renal marker, since kidney function was not evaluated properly.

In the second part of this thesis, we investigated if sCysC was influenced by age, breed, sex and food in order to determine sampling conditions and if separate RIs were required. Serum CysC was not influenced by the different biological factors. This was an advantage over sCr. However, it was of major concern that the majority of cats with CKD of previous studies fell within the RI we determined.

In the third part of the thesis, we partially investigated specificity of CysC, by observing the effect of FIV and hyperthyroidism on sCysC and uCysC. Serum CysC was increased in cats with hyperthyroidism, in contrast with sCr, but not in cats with FIV. Many hyperthyroid cats, but only four cats with FIV had an elevated uCysC/uCr ratio.

In the final study, we evaluated CysC as marker for CKD by comparing sCysC and uCysC between CKD and healthy cats, by correlating GFR with sCysC and sCr and by calculating sensitivity and specificity for detecting decreased GFR. Serum CysC could not distinguish CKD from healthy cats, nor normal from borderline or low GFR in contrast to sCr. Serum CysC also had a lower sensitivity to detect decreased GFR than sCr. Therefore, we conclude that sCysC is not a valuable marker to detect feline CKD. Urinary CysC could not be detected in all CKD cats. Further studies with renal biopsies are needed to further

investigate the possible use of uCysC. This thesis has validated CysC thoroughly at analytical, biological and clinical level.
Despite the fact that we observed a few advantages over sCr, we do not advise to measure CysC for the detection of feline chronic kidney disease with the currently available human nephelometric and turbidimetric assay. Other renal markers should be studied and the influence of early diagnosis on the prognosis of CKD should be investigated.

Despite good analytical and biological validation results, the clinical validation results for sCysC were inadequate. This thesis emphasized how important a solid and complete validation is.

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SUMMARY

Chronic kidney disease (CKD) is a common progressive and irreversible disease in cats. Early diagnosis is important to slow down disease progression. The best method to evaluate kidney function, is estimation of glomerular filtration rate (GFR). However, this method is time-consuming and labour-intensive, and therefore not routinely used. The indirect GFR-markers, serum creatinine (sCr) and urea, are routinely measured, but are insensitive and unspecific. Therefore, markers that can easily and rapidly detect kidney dysfunction in an early stage are urgently needed. A possible marker is cystatin C (CysC). Serum cystatin C (sCysC) fulfills many properties ideal for a GFR marker. Studies in humans and dogs have shown its superiority over sCr, but studies in cats were lacking. In addition, urinary CysC (uCysC) can be used as a marker for proximal tubular damage.

In **Chapter I**, the anatomy and physiology of the kidneys were explained, to better understand the pathophysiology of feline CKD. In this chapter, current knowledge on CysC in human and veterinary medicine was summarized, followed by a brief overview of the principles of appropriate validation. We concluded that CysC has the potential to become a valuable renal marker, but several studies were required. This led us to the objectives of this thesis **(Chapter II)**: the analytical, biological and clinical validation of feline CysC.

At the start of this thesis, no veterinary assays and no analytical validation reports were available on feline CysC. In **Chapter III**, the human particle-enhanced nephelometric (PENIA) and turbidimetric (PETIA) assay were validated, followed by a pilot study with CysC measurement in serum and urine in both CKD and healthy cats. To know which proportion of feline CysC was detected with these human assays, we aimed to produce feline recombinant CysC. Therefore the feline CysC gene (CST3) was isolated from white blood cells and the plasmid with CST3 was cloned into 3 different expression vectors: *Escherichia coli*, *Pichia pastoris* and HEK293T mammalian cells. However, feline recombinant CysC could not be produced. Therefore, the obtained feline CysC concentrations are relative. Both PENIA and PETIA are based on antigen-antibody reaction. Hence, a Western blot analysis was required to demonstrate cross reactivity between feline CysC in serum and urine, and the antibodies from the assays. Bands could not be detected in serum at the expected MM of 13 kDa, but at 26 kDa and 52 kDa. We assumed that polymer formation was caused by denaturing agents. However, the absence of a band in serum could not be readily explained. We hypothesized that this was due to difference in epitope sequence between feline and human CysC. PENIA showed a good precision, linearity and good sensitivity, both in serum

and urine. PETIA had a high LOD, impeding uCysC measurement and was somewhat less accurate. Based on these observations, but also due to practical reasons, we used PENIA for further measurement of feline CysC. The pilot study demonstrated that cats with CKD had a significantly higher sCysC and uCysC/uCr ratio compared with healthy cats. However, an overlap in sCysC could be observed. Since no GFR was determined, the reason for overlap was unclear at that time.

In the second part of this thesis, feline CysC was validated biologically. In **Chapter IV, §4.1**, we evaluated which sample storage conditions were appropriate, if it was mandatory to fasten cats prior to sCysC evaluation and if a diurnal variation could be observed. We demonstrated that samples can be stored at room temperature (RT) if sCysC is analysed within 24 h and at 4 °C for 1 week. If longer storage is required, we advise storage at -20 °C or -72 °C for up to 5 months. Longer storage is not recommended. One freeze-thaw cycle is allowed. Caution is warranted if pCysC has to be compared with sCysC, since pCysC results were significantly lower. We evaluated the effect of feeding versus fasting on both sCr and sCysC in a crossover study with 10 healthy laboratory cats. We observed no significant difference in sCysC and sCr between fasted and fed cats. A significant difference between morning and evening samples could be observed for sCysC, but the differences were only small, and not clinically relevant. In addition, we observed large discrepancies between the individual cats. Based on our findings, it is not mandatory to fasten cats prior to feline sCysC or sCr evaluation.

After our encouraging findings in the pilot study of **Chapter III,** it was essential to determine a reference interval (RI) in a healthy cat population **(Chapter IV, §4.2).** One hundred and thirty cats of different ages, breeds and sexes were included. First we evaluated if there was an influence of age, breed, and sex on feline sCysC, in order to determine if separate RIs were necessary. The routinely measured renal parameters, sCr, urea, SBP, USG and UPC, were also compared between the different groups. We could not observe an effect of age, breed and sex on feline sCysC, in contrast with sCr. Significantly higher sCr concentrations were measured in geriatric cats, compared to young cats. Interestingly, systolic blood pressure (SBP) was significantly higher in geriatric cats compared to middle-aged and young cats; UPC was significantly higher in male intact cats compared to female intact and neutered cats; and urea was influenced by age and gender. However, longitudinal studies should be performed to further elucidate those observations. Since no separate RI for sCysC

was required, we determined a RI for sCysC according to the ASVCP guidelines of 0.58–1.95 mg/L. In addition, we also established a RI for sCr of 64.5–161.8 µmol/L, analysed with the modified Jaffe method, that corresponded with previously published RI with the same method.

It was described in humans that HIV and hyperthyroidism may cause increased sCysC concentrations. In **Chapter V**, **§5.1,** we investigated the influence of hyperthyroidism and FIV on both sCysC and uCysC. This is important information for use in practice. We measured sCysC and uCysC in cats with FIV, hyperthyroid and healthy cats. Also sCr, urea, USG, UPC and TT4 were measured in those cats. In hyperthyroid cats, we expected lower sCr and lower sCysC, since hyperthyroidism leads to increased GFR. However, we observed a significantly higher sCysC and uCysC/uCr compared with healthy cats. Serum Cr was significantly lower, as expected. We also observed an overlap in sCysC between the healthy and hyperthyroid cats and not all hyperthyroid cats had a detectable uCysC concentration. Our findings suggested that sCysC is not a reliable GFR marker in hyperthyroid cats, as previously reported. Additional studies with renal biopsies are needed to evaluate uCysC as tubular marker in hyperthyroid cats. In the FIV-positive group, we did not observe a significantly higher sCysC concentration, and only 4/30 cats had uCysC > LOD. These results could have been influenced by an effect of non-steroidal anti-inflammatory drugs and antibiotics on renal function. Interestingly, in the three groups, there was no association between sCysC and sCr, indicating that non-renal factors, mild kidney dysfunction might have influenced our results or that CysC is not a good GFR marker.

In the final study of this thesis **(Chapter V, §5.1)**, we evaluated the value of CysC for the detection of feline CKD by comparing sCysC and uCysC between CKD and healthy cats, by correlating sCysC and sCr with GFR and by calculating sensitivity and specificity for decreased GFR. GFR was measured with PEC-ICT in 17 CKD and 15 healthy cats. No significantly better correlation between sCysC and GFR compared to sCr was observed for the three methods (Cr, endo-and exo-iohexol). Serum CysC was significantly higher in cats with CKD (n = 49) compared with the healthy cats (n = 41), but an overlap was observed. Therefore, we classified the cats in having normal, borderline or low GFR based on PexICT. Serum CysC overlapped between the three groups, in contrast with sCr, indicating that sCysC is not able to distinguish between those three groups. The sensitivity of sCysC to detect decreased GFR was only 22% compared to 83% for sCr. For uCysC, 15/49 cats with CKD

had uCysC < LOD. Nevertheless tubulo-interstitial lesions are most commonly observed in cats with CKD, glomerular lesions could not be excluded. Additional studies with renal biopsy are warranted. This important study demonstrated that feline CysC measured with the available human assays is not a reliable marker for kidney function evaluation in cats. Therefore, we do not recommend to use this marker in cats.

This thesis provided us new insights in the use of CysC for the detection of feline CKD. In contrast to studies in dogs, we have validated feline CysC at analytical, biological and clinical level. We have shown that the human PENIA and PETIA are able to measure feline CysC, but PENIA is the preferred method. Cystatin C favored sCr at biological level, since sCysC was less influenced by breed, age, and sex. However, during this thesis, we observed that sCysC overlapped between healthy cats and cats with CKD, which was a major concern. Our suspicion was confirmed by comparing the correlation of GFR with sCysC and sCr and by comparing sCysC in cats with normal, borderline and low GFR. Therefore, we can conclude that sCysC measured with the human PENIA or PETIA is not a reliable GFR marker in cats. Urinary CysC should be evaluated more in-depth.

SAMENVATTING

Chronische nierziekte (CNZ) is een vaak voorkomende irreversibele en progressieve aandoening bij katten. Een vroege diagnose is belangrijk om de ziekte af te remmen. De beste methode om de nierfunctie te evalueren, is schatting van de glomerulaire filtratiesnelheid (GFS). Deze methode is echter tijdrovend en arbeidsintensief, en wordt dus niet vaak gebruikt. Bijgevolg worden meestal de indirecte GFS-merkers, serum creatinine (sCr) en ureum gemeten, maar deze zijn niet gevoelig en niet specifiek. Daarom is er behoefte aan merkers die nierdysfunctie eenvoudig en snel kunnen detecteren in een vroeg stadium. Een mogelijke merker is cystatine C (CysC). Serum cystatine C (sCysC) bezit vele eigenschappen van ideale GFS-merker. Studies in de mens en hond hebben de superioriteit van CysC aangetoond boven Cr. Studies bij katten ontbreken echter nog. Bovendien kan urinair CysC (uCysC) gebruikt worden als merker voor schade in de proximale tubulus.

In **Hoofdstuk I** werd de anatomie en fysiologie van de nieren uitgelegd, om beter de pathofysiologie van CNZ te begrijpen. In dit hoofdstuk werd een overzicht gegeven van de huidige kennis over CysC in de humane geneeskunde en diergeneeskunde, gevolgd door een kort overzicht van de principes van validatie. We concludeerden dat CysC veel potentieel had om een waardevolle renale merker te worden, maar dat verschillende studies vereist waren. Dit leidde tot de doelstellingen van deze thesis **(Hoofdstuk II)**: de analytische, biologische en klinische validatie van felien CysC.

Bij de start van dit onderzoek waren geen diergeneeskundige assays beschikbaar, en er waren geen rapporten beschikbaar over analytische validatie van felien CysC. In **Hoofdstuk III** werden de humane partikel-versterkte nefelometrische assay (PENIA) en turbidimetrische assay (PETIA) gevalideerd, gevolgd door een kleine pilootstudie waarbij CysC gemeten werd in serum en urine van zowel katten met CNZ als gezonde katten. Om na te gaan welke proportie felien CysC gedetecteerd kon worden met de humane assays, hadden we de intentie om felien recombinant CysC te produceren. Hiervoor werd het feliene CysC gen (CST3) in drie verschillende expressie-vectors gekloond: *Escherichia coli*, *Pichia pastoris* en HEK293T zoogdiercellen. Felien recombinant CysC kon echter niet geproduceerd worden. Daarom moeten we ervan uitgaan dat met de humane assays relatieve CysC concentraties gemeten worden. Zowel PENIA als PETIA zijn gebaseerd op antigen-antistof reactie. Bijgevolg was een Western blot analyse nodig om kruisreactiviteit aan te tonen tussen het feliene CysC in serum en urine, en de antistoffen van de assays. Er werden geen bandjes gezien ter hoogte van de verwachte 13 kDa MM, maar wel ter hoogte van 26 kDa en 52 kDa. De polymeervorming

kon veroorzaakt zijn door het gebruik van denaturerende agentia. De afwezigheid van een band in serum kon echter niet onmiddellijk verklaard worden. Onze hypothese was dat dit te wijten was aan een verschil in epitoopsequentie tussen het feliene en humane CysC. PETIA vertoonde een hogere detectielimiet, wat meting van uCysC zou kunnen bemoeilijken, en was ook iets minder accuraat dan de PENIA. Gebaseerd op de validatieresultaten, en uit praktische overwegingen, gebruikten we de PENIA voor de verdere analyses van felien CysC. Onze pilootstudie toonde aan dat katten met CNZ een significant hogere sCysC en uCysC/uCr ratio hadden vergeleken met de controlekatten. Er kon echter wel een overlap worden gezien in de sCysC concentratie. Aangezien er geen GFS gemeten was, kon de reden voor overlap op dit moment niet verklaard worden.

In het tweede deel van deze thesis werd CysC biologisch gevalideerd. In **Hoofdstuk IV, §4.1,** evalueerden we hoe stalen bewaard kunnen worden en of het noodzakelijk was dat katten moesten vasten voor sCysC evaluatie en of er een diurnale variatie optrad. We toonden aan dat stalen bewaard kunnen worden op kamertemperatuur indien sCysC geanalyseerd wordt binnen de 24 u en op 4 °C gedurende 1 week. Als de stalen langer bewaard moeten worden, adviseren we bewaring op -20 °C of -72 °C tot 5 maanden. Langere bewaring raden we niet aan. Aandacht is vereist als pCysC vergeleken moet worden met sCysC, aangezien de pCysC concentraties significant lager waren. We evalueerden het effect van voeding en vasten zowel op sCysC als sCr in een crossover studie met 10 gezonde proefdierkatten. We observeerden geen verschil in sCysC en sCr tussen katten die nuchter waren en katten die gegeten hadden. Een significant verschil in sCysC werd geobserveerd tussen de ochtend- en avond stalen, maar de verschillen waren klein en niet klinisch relevant. Daarnaast werden grote verschillen tussen de individuele katten waargenomen. Gebaseerd op onze bevindingen, is het niet noodzakelijk om katten te laten vasten voor evaluatie van felien sCysC of sCr.

Na de veelbelovende bevindingen in de pilootstudie van **Hoofdstuk III**, was het essentieel om een referentie interval (RI) te bepalen in een gezonde kattenpopulatie **(Hoofdstuk IV, §4.2).** Honderddertig katten van verschillende leeftijden, geslacht en ras werden geïncludeerd. Eerst evalueerden we of er een invloed was van deze fysiologische factoren, om na te gaan of verschillende RI nodig waren. De routine renale merkers, sCr, ureum, de systolische bloeddruk (SBP), het urinair soortelijk gewicht (USG) en de urinaire eiwit:creatinine ratio (UPC) werden ook geëvalueerd. We konden geen invloed van geslacht, leeftijd en ras op felien sCysC observeren, in tegenstelling tot sCr. Er werden significant

hogere sCr concentraties gemeten bij de geriatrische katten, vergeleken met de jonge katten. De SBP was significant hoger in geriatrische katten vergeleken met katten van jonge en middelbare leeftijd; de UPC was significant hoger in mannelijke katten vergeleken met de vrouwelijk intacte en gesteriliseerde katten; en ureum was beïnvloed door leeftijd en geslacht. Er zouden echter longitudinale studies moeten worden uitgevoerd om deze bevindingen verder te onderzoeken. Aangezien geen aparte RI nodig waren, werd een RI van 0.58–1.95 mg/L bepaald voor sCysC volgens de ASVCP richtlijnen. Het was echter wat zorgbarend dat de sCysC concentratie van de meeste CNZ katten uit voorgaande studies binnen ons RI viel. Dit moedigde bijkomende studies aan. Bovendien bepaalden we in deze studie ook een RI voor sCr, gemeten met de gemodificeerde Jaffe methode, van 64.5–161.8 µmol/L dat overeenkwam met eerder gepubliceerde RIs voor sCr bepaald met dezelfde methode.

Publicaties uit de humane geneeskunde toonden aan dat HIV en hyperthyroïdie een stijging van sCysC concentraties kunnen veroorzaken. In **Hoofdstuk V**, **§5.1,** onderzochten we de invloed van FIV en hyperthyroïdie op zowel sCysC als uCysC. Dit is belangrijke informatie voor gebruik in de praktijk. We bepaalden sCysC en uCysC in katten met FIV, katten met hyperthyroïdie en gezonde katten. Ook werden sCr, ureum, USG, UPC en totaal thyroxine bepaald. We verwachtten lage sCr en sCysC concentraties bij de hyperthyroïde katten, aangezien hyperthyroïdie een gestegen GFS veroorzaakt. We observeerden echter een significant hogere sCysC concentratie en uCysC/uCr ratio vergeleken met de gezonde katten. Serum Cr was significant lager, zoals verwacht. We observeerden ook een overlap in sCysC tussen de gezonde en hyperthyroïde katten en niet alle hyperthyroïde katten hadden detecteerbare uCysC. Onze bevindingen toonden aan dat sCysC geen goede GFS merker is bij hyperthyroïde katten, zoals ook eerder beschreven. Er zijn echter bijkomende studies nodig met nierbiopsie om uCysC te evalueren als tubulaire merker bij hyperthyroïde katten. In de FIV-positieve katten konden we geen significant hogere sCysC concentratie waarnemen en uCysC kon slechts bij 4/30 katten geobserveerd worden. Bovendien zouden deze resultaten beïnvloed kunnen zijn door een effect van niet-steroïdale anti-inflammatoire geneesmiddelen en antibiotica op de nierfunctie. In de drie groepen was er geen associatie tussen sCysC en sCr, wat betekent dat niet-renale factoren, milde nierdysfunctie onze resultaten beïnvloed kon hebben, of dat sCysC geen goede GFS-merker is.

In de laatste studie van deze thesis **(Hoofdstuk V, §5.2)**, hebben we nagegaan of CysC CNZ kan detecteren in katten, door sCysC en uCysC te vergelijken tussen katten met CNZ en

gezonde katten, door sCysC en sCr te correleren met GFS en de sensitiviteit en specificiteit van sCysC en sCr te berekenen om afgenomen GFS vast te stellen. De GFS werd geëvalueerd aan de hand van de gecombineerde plasma exogene creatinine-iohexol klaringstest (PEC-ICT) in 17 katten met CNZ en 15 gezonde katten. Er werd geen significant betere correlatie gevonden tussen sCysC en GFS vergeleken met sCr voor geen enkele van de drie geteste methoden (exogeen creatinine, endo- en exo-iohexol). Serum CysC was significant hoger bij katten met CNZ (n = 49) vergeleken met de gezonde katten (n = 41), maar een overlap kon geobserveerd worden. Gebaseerd op de resultaten van de plasma exo-iohexol klaringstest (PexICT), werden katten geclassificeerd met een lage, borderline of normale GFS. Serum CysC overlapte tussen deze groepen, in tegenstelling tot sCr. De sensitiviteit van sCysC om gedaalde GFS te detecteren was slechts 22% vergeleken met 83% voor sCr. De uCysC concentratie lag beneden de detectielimiet in 15/49 katten met CNZ. Niettegenstaande katten met CNZ vooral tubulo-interstitiële letsels vertonen, konden glomerulaire letsels niet uitgesloten worden. Bijkomende studies met nierbiopsie zijn vereist. Deze belangrijke studie toonde aan dat felien sCysC geen betrouwbare merker is om de nierfunctie te evalueren bij de kat. Daarom raden het gebruik van deze merker af in de feliene geneeskunde.

Deze thesis gaf ons nieuwe inzichten in het gebruik van CysC voor de detectie van CNZ bij katten. In tegenstelling tot studies bij de hond, hebben we een grondige validatie uitgevoerd van felien CysC op analytisch, biologisch en klinisch niveau. We hebben aangetoond dat de humane PENIA en PETIA felien CysC kunnen meten, maar de PENIA geniet de voorkeur. CysC was minder beïnvloed door biologische factoren dan sCr. Tijdens de verschillende studies bemerkten we echter een overlap in sCysC tussen de katten met CNZ en de gezonde katten. Onze waarnemingen werden bevestigd door de GFS te correleren met sCysC en sCr en door sCysC te vergelijken in katten met normale, borderline en lage GFS. Daarom kunnen we concluderen dat sCysC gemeten met de humane PENIA en PETIA geen betrouwbare merker is voor GFS bepaling bij de kat. Urinair CysC zou nog verder moeten onderzocht worden.

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CURRICULUM VITAE

Liesbeth Ghys werd geboren op 2 augustus 1986 in Oostende. In 2004 behaalde ze haar diploma secundair onderwijs in de richting Wetenschappen-Wiskunde aan het Onze-Lieve-Vrouwecollege in Oostende. Onmiddellijk daarna startte ze haar studie Diergeneeskunde aan de Universiteit Gent. In 2010 behaalde ze met grote onderscheiding het diploma van Dierenarts in de afstudeerrichting Gezelschapsdieren.

Geboeid door het wetenschappelijk onderzoek startte ze in 2010 met een doctoraatsstudie over nierfunctie bij katten. Deze studie werd gefinancierd door de Facultaire Onderzoekscommissie van de Faculteit Diergeneeskunde en het Fonds voor Innovatie door Wetenschap en Technologie in Vlaanderen (IWT). Tevens behaalde ze in 2015 het diploma van de Doctoraatsopleiding in de Diergeneeskundige Wetenschappen.

Liesbeth Ghys is auteur of co-auteur van verschillende wetenschappelijke publicaties en abstracts. Zij nam deel aan verschillende nationale en internationale congressen.

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