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Novel biomarker in cats affected with spontaneous CKD: focus on hypertension and proteinuria

Tutor: Professor Paola Scarpa

Dott. Marco GIRALDI
Matr. R 10953

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Abstract

Chronic kidney disease (CKD) is a common disease of elderly cats characterized by a progressive course and a high mortality rate. The development and the progression of CKD depend on the presence of complications such as proteinuria and hypertension. Hence, a correct staging of the disease based to these latter pathologic states together with serum creatinine, as suggested by the International Renal Interest Society (IRIS) guidelines, is recommended to properly treat affected cats. In this regard, hypertension is often difficult to confirm given the high frequency of white-coat hypertension in this specie. Moreover, although decisive progresses have been achieved in the last years, an early diagnosis of CKD or of hypertension and proteinuria associated with CKD is still challenging in cats. Early identification of ongoing kidney injury may allow veterinarians to prevent worsening of the disease and ultimately to improve the quality of life of affected feline patients.

The first part of this thesis was focused on a preliminary evaluation of preanalytical and analytical variability of parameters used in IRIS staging of CKD: preanalytical and analytical variability of urinary protein-to-creatinine (UPC) ratio (study 1); the effect of hematuria and pyuria on UPC (study 2); and the physiologic concentration of the novel biomarker of glomerular filtration rate symmetric dimethylarginine (SDMA) in Holy Birman cats (study 3).

The second main part of this thesis was aimed to assess how biomarkers such as serum big endothelin-1 (big-ET1), homocysteine (Hcy), aldosterone and urinary big-ET1, alpha-1 microglobulin (A1MG) and the presence of tubular proteins in urine (evaluated with sodium dodecyl sulfate-agarose gel electrophoresis, SDS-AGE) may allow an early diagnosis of CKD, may identify cats at risk of severe worsening of the disease, or may be a predictor of hypertension (study 4). To this aim, privately owned cats (i.e. clinically healthy cats at risk to develop CKD and cats affected with CKD) were prospectively enrolled and sampled over time.

The results of study 1 demonstrated that proteinuria was stable up to to 6 hours at room temperature, 1 week at refrigeration temperature, four freeze-thaw cycles and 4 weeks at −20°C, whereas the use of different analytical methods resulted in inaccuracy and suboptimal concordance in classifying samples according to IRIS substaging, that in turn can potentially affect clinical decisions, make questionable the comparison of UPC results between different laboratories, and have significant impact in substaging cats affected by

CKD. Study 2 showed that hematuria could affect proteinuria, suggesting that diagnosis and staging of renal proteinuria should be avoided or interpreted with caution in samples with high hematuria. Study 3 highlighted that SDMA may be a better marker of CKD than creatinine in Birman cats and the analysis of both creatinine and SDMA could help prevent errors in diagnosing and staging CKD in Birman cats.

In study 4, different results were found with the different biomarkers. Big-ET1 did not give satisfying results in serum, whereas in urine the ELISA method yielded satisfying validation results, supporting its introduction in this specie. Urinary big-ET1 was associated with the severity of CKD and proteinuria, revealing that it could be a promising aid in nephropatic cats and could shed light on the pathogenesis of tubulo-interstitial and glomerular damage in cats with CKD. The method to measure Hcy can be considered reliable in cats according to the validation tests. Serum Hcy increased progressively with the progressive increase in severity of CKD and the detection of high Hcy in some non-azotemic patients with CKD could add this new marker to those currently available for the identification and staging of the kidney disease of the cat. Conversely, no direct relationship was found between Hcy and hypertension. Aldosterone was not associated with severity of CKD, proteinuria and SBP. Therefore, neither Hcy nor aldosterone can be considered an indicator of hypertension in cats affected with CKD. The ELISA kit used for A1MG measurement failed all the validation tests and results were considered unacceptable; further studies are therefore needed to investigate the presence of A1MG in cats with CKD and tubular damage. SDS-AGE showed the consistent presence of bands with high molecular weight in healthy cats, suggesting that this pattern is normal in this specie and has to be taken into account while evaluating proteinuria in cats; tubular bands were frequent in patients with CKD at any stage, confirming the predominant tubule-interstitial damage of this disease in cats and suggesting that SDS-AGE can be considered a valuable aid in diagnostic approach to feline CKD.

Abstract (Italian version)

La patologia renale cronica (CKD) è una malattia comune nei gatti anziani, caratterizzata da un decorso progressivo e da un elevato tasso di mortalità. Lo sviluppo e la progressione della CKD dipendono dalla presenza di fattori complicanti, quali proteinuria e ipertensione. Pertanto, per una corretta gestione terapeutica, è necessario stadiare la patologia in base a questi due stati patologici, contestualmente ai livelli di creatinina sierica, come raccomandato dalle linee guida della International Renal Interest Society (IRIS). A questo proposito, nel gatto l'ipertensione è spesso difficile da confermare data l'elevata frequenza dell'ipertensione da "camice bianco".

Inoltre, anche se negli ultimi anni sono stati conseguiti importanti progressi nell'approccio diagnostico della CKD felina, la diagnosi precoce di CKD o di ipertensione e proteinuria associate alla CKD è ancora problematica in questa specie. L'identificazione precoce del danno renale cronico attivo potrebbe consentire al veterinario di attuare gli accorgimenti terapeutici necessari a rallentare la progressione della malattia e, in ultima analisi, migliorare la qualità della vita dei pazienti nefropatici felini.

La prima parte di questa tesi si è focalizzata sulla valutazione preliminare della variabilità preanalitica e analitica di alcuni parametri utilizzati nella stadiazione del gatto nefropatico: la variabilità preanalitica e analitica del rapporto proteine urinarie su creatinina urinaria (UPC) (studio 1); l'effetto dell'ematuria e della piuria sulla UPC (studio 2); e la concentrazione fisiologica del nuovo biomarker di filtrazione glomerulare dimetilarginina simmetrica (SDMA) nei gatti Holy Birman (studio 3).

La seconda parte di questa tesi si è orientata sulla valutazione dell'utilità di biomarker sierici e urinari nella diagnosi precoce di CKD, nella identificazione dei gatti a rischio di peggioramento della malattia, e nell'identificazione dell'ipertensione (studio 4). In particolare sono stati presi in esame big endothelin-1 (big-ET1), omocisteina (Hcy) e aldosterone nel siero, e big-ET1, alfa-1 microglobulina (A1MG) e bande di proteine tubulari (rilevate con sodium dodecyl sulfate-agarose gel electrophoresis ,SDS-AGE) nelle urine. A tal fine, gatti clinicamente sani a rischio di sviluppare CKD e gatti affetti da CKD sono stati inseriti nello studio prospettivamente e campionati nel tempo.

I risultati dello studio 1 hanno dimostrato che la proteinuria è stabile fino a 6 ore a temperatura ambiente, fino 1 settimana a temperature di refrigerazione, dopo 4 cicli di congelamento-disgelo e fino 4 settimane a -20° C, mentre l'utilizzo di diversi metodi analitici ha evidenziato la mancata accuratezza e la concordanza non ottimale nella classificazione dei pazienti secondo la stadiazione IRIS, suggerendo come queste

variabilità possano potenzialmente influenzare le decisioni cliniche, rendere discutibile il confronto dei risultati di UPC tra diversi laboratori, e incidere in modo significativo sulla suddivisione in stadi dei gatti colpiti da CKD. Lo studio 2 ha mostrato che l'ematuria potrebbe influenzare la proteinuria, indicando che la diagnosi e la stadiazione della proteinuria renale dovrebbero essere evitati o interpretati con cautela nei campioni con ematuria elevata. Lo studio 3 ha evidenziato che SDMA potrebbe essere un biomarker di CKD migliore rispetto alla creatinina nei gatti di Birmania e l'analisi contemporanea di creatinina e SDMA potrebbe aiutare a prevenire gli errori nella diagnosi e nella stadiazione di CKD nei gatti di Birmania.

Nello studio 4, la big-ET1 non ha dato risultati soddisfacenti nel siero, mentre nell'urina con il metodo ELISA sono stati ottenuti buoni risultati di validazione, supportando la sua applicazione in questa specie animale. Inoltre, la big-ET1 urinaria è risultata essere associata alla gravità della CKD e della proteinuria, rivelando una possibile utilità in ambito diagnostico in gatti nefropatici e gettando luce sulla patogenesi della nefropatia tubulo-interstiziali e dei danno glomerulare nei gatti con CKD. Considerati i buoni risultati della validazione, anche il metodo per la misurazione della Hcy potrebbe essere considerato affidabile nei gatti. Dato l'incremento progressivo della concentrazione di Hcy con l'aggravarsi della nefropatia e la presenza di alcuni pazienti non iperazotemici con Hcy elevata evidenziati nel nostro studio, questo nuovo biomarker potrebbe aggiungersi a quelli attualmente disponibili per l'identificazione e la stadiazione della malattia renale. Nonostante questi buoni risultati, la relazione tra la Hcy e ipertensione è risultata assente.

L'aldosterone non è risultato associato alla gravità di CKD, proteinuria e ipertensione. Pertanto, l'aldosterone sierico, come la Hcy sierica, non sembrano essere utili nell'indicare presenza di ipertensione nei gatti colpiti da CKD. Il kit ELISA utilizzato per la quantificazione urinaria di A1MG ha fallito tutti i test di validazione e, pertanto, i risultati ottenuti sono stati considerati inattendibili, suggerendo ulteriori studi per esaminare la presenza di A1MG nei gatti con CKD e danno tubulare. La SDS-AGE ha mostrato la presenza di bande ad alto peso molecolare nei gatti sani, indicando che la loro presenza sia normale in questa specie ed esortando a tenere in considerazione questo pattern nella valutazione della proteinuria nei gatti; inoltre, le bande tubulari sono risultate frequenti in pazienti nefropatici a tutti gli stadi, confermando il prevalente danno tubulo-interstiziale nella CKD felina e suggerendo che l'SDS-AGE può essere considerata un valido aiuto nell'approccio diagnostico di questa patologia.

Introduction

Chronic kidney disease (CKD) is usually defined as structural and/or functional abnormalities of one or both kidneys that have been present for a period of 2-3 months or longer (Reynolds and Lefebvre, 2013).

CKD is common in cats and the prevalence increases with age. Early studies suggested that up to 15% to 30% of cats older than 15 years of age show evidence of CKD (Reynolds and Lefebvre, 2013) but more recent studies suggest that the prevalence of CKD is much higher, estimating that 80% of cats greater than 15 years old may have renal impairment (Jepson, 2016).

Irrespective of the different triggering causes, CKD is characterized by the progressive and irreversible loss of nephrons. The rate of progression could be variable and depends on many factors but it is generally slow and not common (Chakrabarti et al., 2012).

The current view of the pathogenesis of CKD is that one (or more) initiation factor(s) precedes a phase in which progression factors perpetuate the kidney injury (Brown et al., 2016).

Among the initiation factors, the well-defined diseases that can trigger CKD are:

- Amyloidosis
- Renal dysplasia
- Polycystic kidney disease
- Pyelonephritis (bacterial)
- Nephrolithiasis
- Chronic viral infections: feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), feline infection peritonitis (FIP), feline morbillivirus (FmoPV)
- Immune complex glomerulonephritis
- Acute kidney injury
- Chronic feeding of unbalanced diets
- Lymphoma

These diseases could initiate CKD by injuring feline kidney in different ways, some localizing to a particular region of the kidney and some affecting the whole nephron and kidney, and the specific mechanisms are discussed elsewhere (*).

* Glick et al., 1978; Boyce et al., 1984; DiBartola et al., 1987; DiBartola et al., 1993; Lyons et al., 2004; Kyles et al., 2005; Aresu et al., 2009; Baxter et al., 2012; Poli et al., 2012; Pedersen, 2014, Sieg et al., 2015

When one of these diseases is diagnosed concurrently to the chronic renal impairment, it could be considered the cause of CKD. Conversely to human and dogs, in the majority of cats with CKD a specific primary disease could not be identified and the nephropathy is therefore termed idiopathic. In these cases, it seems likely that a combination of intrinsic factors, repeated acute insults and environmental factors act synergistically as initiating factors. The recognized and suspected initiation factors are: ageing, comorbidities, AKI, vaccinations, stress and chronic hypoxia (Brown et al., 2016). There are no evidences that these factors alone could initiate CKD, therefore it is currently accepted that only the combination of two or more of these factors and their persistent and repeated action over time during the life explain the development of the form of CKD that is recognized as idiopathic. These factors are discussed in an excellent recent review (Brown et al. 2016).

The main pathological feature of feline CKD, especially idiopathic CKD, is the chronic tubule-interstitial inflammation. Inflammatory infiltrate is mainly represented by lymphocytes that are present at any stage of the disease (McLeland et al., 2015).
Interstitial fibrosis and scarring occur as consequence of chronic cellular injuries and inflammatory stimuli and it's considered an irreversible damage of the kidney. Collagen deposition correlates with the degree of CKD and is the lesion that best correlated with severity of azotemia (Chakrabarti et al., 2012).

A plethora of other pathologic alteration affecting other part of the kidney and the nephron could occur during the progression of disease: tubular degeneration comes together to interstitial inflammation and fibrosis, and is more severe in later stages; also glomerulosclerosis progressively worsened with stage progression but it's deemed secondary to adaptive changes due to the loss of nephrons; vascular alterations such as hyperplastic arteriolosclerosis, fibrointimal hyperplasia, or other lesions could be present (McLeland et al., 2015).

Beyond the different mechanisms that are able to trigger the kidney injury, the progressive loss of nephrons lead to numerous sequelae that self-perpetuate the chronic kidney injury and/or are able to cause other systemic injuries by its self. The progression factors are: proteinuria, hypertension (systemic and glomerular), renin-angiotension-aldosterone system activation, phosphorous intake, sodium intake, anemia and tubular hypoxia.

Azotemia, electrolyte abnormalities, acid-base disorders (metabolic acidosis), dehydration, loss of muscle mass are other common

Most of them are also prognostic factors and they need to be investigated and specific treatment instituted in order to slow the progression of CKD. Azotemia, proteinuria and hypertension are discussed below.

Azotemia. The progressive structural and functional loss of the nephrons leads to a global reduction of the glomerular filtration rate (GFR). The retention of endogenous substances that otherwise are filtered and excreted by the kidney is the direct effect of the decreased GFR. Among these substances (mainly between 500 and 12.000 KDa) are present amino acids, nitrogenous substances, cytokines and hormones and other molecules that are the final metabolites of various metabolic pathways.

Their increasing concentration leads to direct or indirect toxic effect on different cells types and organs and causes the clinical appearance of a group of signs defined “uremic syndrome”. It is generally accepted that clinical signs associated to azotemia occur when at least 75% of functional mass is lost (Brown et al., 1997). However, a recent study found that some mildly azotemic cats had only 25-50% of altered parenchyma, pointing out that even a mild degree of lesions (less than 75%) could have functional significance (McLeland et al., 2015).

Proteinuria. In CKD, proteins could be classically lost in urine by the increased amount of filtered protein by the injured glomerulus and/or by the impairment reabsorption from tubular cells.

Proteinuria can contribute directly to chronic kidney injury mainly in case of primary glomerular injury. In this scenario, tubular cells are overloaded by the proteins and as a consequence, can degenerate, degrade for apoptosis and secrete inflammatory cytokines, contributing to and accelerating chronic interstitial inflammation (D'amico and Bazzi, 2003; Chakrabarti, 2013). Primary glomerular injury is not common in cats and is reported to be the leading cause of CKD in certain kidney disease such as familiar amyloidosis (Boyce et al., 1984) and FIV infection, in which both glomerular amyloidosis and presumed immune complex glomerulonephritis have been described (Poli et al., 2012).

Accordingly, cats with immunodeficiency virus (FIV) had higher proteinuria compared to non–infected cats in two studies (Baxter et al., 2012; Taffin et al., 2017) and moderate to severe proteinuria was found in cats with familiar amyloidosis (White et al., 2008;

Paltrinieri et al., 2015). Primary hypertension is considered an other cause of increase filtration of proteins by glomerulus in human (Obialo et al., 2002) whereas in cats it is unclear whether CKD-associated hypertension contribute to the magnitude of proteinuria or not, since discordant results were found: an earlier study showed that cats with hypertension were more proteinuric than normotensive cats with equivalent stage of CKD (Syme et al., 2006) whereas a recent study found no influence of hypertension on proteinuria in cats (Bijsmans et al., 2017).

The majority of cats with chronic kidney disease are affected by the idiopathic form, and proteinuria is mild and more common at late stages in this case (Syme, 2006), suggesting that it is mainly a consequence rather than a cause of kidney injury. (Chakrabarti et al., 2013) Accordingly, it was associated with increased severity of tubular degeneration, inflammation and fibrosis in a study (McLeland et al., 2015).

Nevertheless persistent proteinuria is a sign of kidney damage and also a strong indicator for progression (Chakrabarti et al., 2012; Lees 2005; Syme 2009; Jepson 2009) and mortality (Syme et al., 2006; King et al., 2007) of CKD. However, although both ACEi and ARB significantly reduce the magnitude of proteinuria, the benefit in terms of slowing progression of CKD and improve survival has yet to be demonstrated (King et al., 2006; Sent et al., 2015).

Hypertension. Systemic hypertension (HT) is considered to be both a cause and a consequence of CKD (Syme, 2011). HT is an important potential complicating factor of CKD (Syme, 2011) and is common in cats with this disease. Depending on the population studied and the cut-off used to define HT, prevalence could range from 19.4% (Syme et al., 2002a) until 61% (Kobayashi et al., 1990). Also, CKD is the most common disease associated with HT and cats (Brown et al., 2007) since approximately 3/4 of HT patients were reported to be also azotemic in a study (Taylor et al., 2017).

The persistence of high SBP can lead to severe clinical consequences due to target organ damage (TOD), affecting the eyes, heart, brain and the kidney itself (Sansom et al., 2004) (Taylor et al., 2017). On the kidney, HT can lead to glomerular lesions such as hyperplastic arteriolosclerosis, fibrointimal hyperplasia, or other vascular lesions (Chakrabarti et al., 2013) but, in contrast to people, in cats are generally mild. On this regard, hyperplastic arteriolosclerosis, a feature of systemic hypertension thought to be due to microvascular injury, was found in 48% of normotensive cats with CKD. This may indicate that systemic blood pressure does not adequately represent local renal

hemodynamics in cats (McLeland et al., 2015). Accordingly, the evidence for systemic hypertension contributing to the development or progression of feline CKD is unclear since SBP was not identified as a risk factor (Syme et al., 2006; Jepson et al., 2009; Chakrabarti et al, 2012). However, in these studies all the cats were treated with antihypertensive drugs and it could be considered a confounding factor.

The mechanisms beyond the regulation of SBP HT is very complex and multiple molecules and receptors are simultaneously involved (Syme, 2011). Therefore, in cats as in other species, no single cause is recognized to be predominant in the pathogenesis of CKD-associated HT (Syme, 2011).

The renin-angiotensin-aldosterone system (RAAS) is particularly important as a modulator of blood pressure and fluid balance. Since the kidney contains all the necessary components for local RAAS activation, systemic activation of this mechanism could not reflect the renal RAAS.

Angiotensin II and aldosterone are the two final effectors of this system. It is generally accepted that during the CKD progression, the loss of nephrons leads to increase renin production by the underperfused nephrons, which in turn cause increase angiotensin II and aldosterone synthesis (hyperaldosteronism) and HT (Syme, 2011).

Nephropathic cats with hypertension actually tend to have higher aldosterone concentration compared to normotensive cats with CKD (Syme et al., 2002b) and healthy cats (Jensen et al., 1997). However, it was shown that renin concentration was normal or also low during CKD (Jensen et al., 1997) and (Hogan et al., 1999). This result was in accordance with another study in which was showed that, in cats with CKD and HT, aldosterone and renin concentrations did not change in response to ACEi (Syme et al., 2002b). Moreover, also SBP was not affected after ACEi and ARB administration (Syme et al., 2002b; Sent et al., 2015).

These results highlights that the activation of the RAAS seems to be less important in the development CKD-associated hyperaldosteronism and HT in this specie. The increase of aldosterone secretion is probably independent (or only partly dependent) by the renin secretion and increased angiotensin II synthesis by injured nephrons.

Interestingly, in a study was shown that some cats presented with signs of hypertension had extensive micronodular hyperplasia of the zona glomerulosa at the histologic examination of their adrenal glands (Javadi et al., 2005). However, no different adrenocortical histopathologic findings were found between hypertensive and

normotensive cats with CKD, although the majority of cats of both groups had adrenocortical hyperplasia (Keele et al., 2009).
Salt intake was show to affect hypertension in human but it seems to have minor effect on hypertension in cats with or without CKD (Luckschander et al., 2004; Buranakarl et al., 2004; Kirk et al., 2006) although in end stage kidney disease the capacity of the kidney to excrete sodium may decrease.

Diagnosis and staging of feline CKD

The diagnostic process in patients suspected to have CKD requires the interpretation and integration of multiple tests, because most of the clinical and laboratory alterations associated with CKD are also typical of many other systemic diseases. Moreover, since the kidney have different functions and could be affected in different part in naturally occurring disease, different pattern of abnormality could occur and a wide panel of test are almost always necessary.
Given the high compensatory capacity of the kidney, clinical signs typically occur when most of the kidney is injured. They have been the subject of a recent review (Bartges, 2012) and most common are anorexia, polyuria, polydipsia, weight loss, lethargy, halitosis, vomit and diarrhea.
Collection and analysis of blood and urinary samples are mandatory to confirm the diagnosis of CKD. The primary goals of the laboratory tests are to evaluate the different renal functions and detect which of them are compromised. The maintenance of the GFR, the capacity to concentrate or dilute the urine and the retention of molecules such as proteins by the glomerulus are the main altered functions during CKD progression. GFR can be measured by injection of exogenous substances such as (inulin, creatinine and iohexol) to detect the rate of reduction in blood, or indirectly estimated by the measurement of endogenous substances excreted only by glomerular filtration such as creatinine and symmetric dimethylarginine (SDMA) (Yerramilli et al., 2016). The capacity to concentrate or dilute the urine is commonly estimated by the evaluation of urine specific gravity (USG) by the means of a refractometer and in cats values lower than 1035 are suggestive of reduction of concentration ability (Reppas and Foster, 2016).
A second goal is to evaluate other complications that could be present at any stage and need specific treatment. The most common are hyperphosphatemia, anemia, metabolic acidosis, dehydration, hypokalemia and hypo/hypercalcemia. As already described above, most of them are also prognostic factors (Bartges, 2012). Excess of protein in urine can be

screened with rapid tests (such as dipstick) and more accurately quantified with wet biochemistry, and suggests glomerular impairment or tubular reabsorption defects (Littman, 2011; Vaden and Elliot, 2016).

A third but not less important goal to perform blood and urine collection and analysis is to stage the disease, in order to properly treat a patient and have an estimation of the prognosis. In the last decade the International Interest Society (IRIS) has published and updated worldwide accepted guidelines to stage feline patients affected by CKD (Elliott and Watson, 2016). The staging has the aim to aid clinicians to facilitate both choice of appropriate treatment and monitoring of the patient. This staging system is based primarily on serum creatinine and, after an update in 2015, on serum SDMA. Moreover, it is also based on proteinuria and SBP as sub-staging. Below these parameter are described and discussed.

Measurement of GFR

CREATININE**.** Creatinine is the product of the nonenzymatic dehydration from creatine and the conversion occurs almost at constant rate (Braun et al., 2003). After the release in blood, creatinine is freely filtered by the glomerulus and no secretion in renal proximal tubules is demonstrated in cats. Therefore, measurement of endogenous serum or plasma creatinine concentration allows an indirect extimation of GFR (Yerramilli et al., 2016).

Is generally accepted that the increase of creatinine over the reference interval of normality occurs when at least 75% of functional mass is lost (Brown et al., 1997)

However, a recent study found that some cats with mild azotemia had only 25-50% of altered parenchyma, pointing out that even a mild degree of lesions could have functional significance (McLeland et al., 2015).

Beyond the kidney function, muscle mass is an important endogenous determinant of the serum creatinine concentration. On this regard, at late stages of CKD sarcopenia could occur. Hence serum creatinine could loss sensitivity for disease progression as the CKD advance. Moreover, some feline breeds, such as Siberian, Holy Birman cats and, to a lesser extent, Siamese and Somali cats, have a physiologically high concentration of serum creatinine (and urea) compared with the feline general population (Reynolds et al., 2010; Paltrinieri et al., 2014). Therefore, the presence of renal disease risks to be overestimated in these breeds.

IRIS staging based on creatinine requires at least two samples collected in two occasions in the stable patient and is shown in Table 1.

Table 1 IRIS staging based on serum creatinine

Stage	Blood creatinine mg/dl	Comments
At risk	<1.6	History suggests the animal is at increased risk of developing CKD in the future because of a number of factors (such as, exposure to nephrotoxic drugs, breed, high prevalence of infectious disease in the area, or old age).
1	<1.6	Nonazotemic. Some other renal abnormality present (such as, inadequate urinary concentrating ability without identifiable nonrenal cause, abnormal renal palpation or renal imaging findings, proteinuria of renal origin, abnormal renal biopsy results, increasing blood creatinine concentrations in samples collected serially).
2	1.6-2.8	Mild renal azotemia (lower end of the range lies within reference ranges for many laboratories, but the insensitivity of creatinine concentration as a screening test means that animals with creatinine values close to the upper reference limit often have excretory failure). Clinical signs usually mild or absent.
3	2.8-5	Moderate renal azotemia. Many extrarenal clinical signs may be present.
4	>5	Increasing risk of systemic clinical signs and uraemic crises

SDMA**.** SDMA is the methylated form of the amino acid arginine, which is released into the circulation during the normal catabolism of body proteins. (Fleck et al., 2003) Similarly to creatinine, SDMA is excreted primarily by the kidneys (>90%) and therefore its serum concentration increases when the glomerular filtration rate decreases, as it occurs during CKD in cats. (Jepson et al., 2008) SDMA is not affected by body mass in dogs (Hall et al., 2015) and it is likely that this occur also in cats.

The increase of CKD in patients affected by CKD seems to be earlier than the increase of creatinine in cats (Hall et al., 2014a). Therefore, SDMA has been proposed as a novel marker for the early diagnosis of CKD and it is recently added to the IRIS Staging Guidelines for CKD (Elliot and Watson, 2016). As for creatinine, re-evaluation within two weeks in stable patients is necessary in case of abnormal results.

IRIS guideline suggests to measure SDMA together to creatinine and, according to results, the refining of the staging or treatment recommendations is carried out, as shown in Table 2.

Table 2 IRIS staging refinement recommendation based on blood SDMA

Creatinine	Blood SDMA	Recommendation
<1.4 mg/dL (dogs) or <1.6 mg/dl (cats)	Persistent SDMA >14 µg/dl	Stage the dog or cat as IRIS CKD Stage 1
IRIS CKD Stage 2 + low BCS	SDMA ≥25 µg/dl	Treatment recommendations listed under IRIS CKD Stage 3 for this patient.
IRIS CKD Stage 3 + low BCS	SDMA ≥45 µg/dl	Treatment recommendations listed under IRIS CKD Stage 4 for this patient.

BCS: body condition score

Measurement of Proteinuria

Proteinuria can be routinely assessed via semi-quantitative methods such as urine dipstick colorimentric test and/or SSA.

Dipsticks (or dry reagent test strips) are rapid and inexpensive methods that allow, along with other urinary physico-chemical or cytological parameters, a first evaluation of the presence or absence of proteins in urine in a point-of-care setting (Stockham and Scott, 2008).

When proteins (mainly albumin) are present, the reaction with the pad yields a variable color change whose intensity is proportional to the protein concentration. Results are then expressed semi-quantitatively, usually as negative, trace or 1+ to 4+ (corresponding, for most of the commercially available dipsticks to 15 to 2000 mg/dL of proteins), comparing the pad against the chart on the side of the dipstick package or, alternatively, loading the strip in an automated spectrophotometric reader.

In cats, the sensitivity and specificity of the dipstick (≥ trace reaction) for albumins in urine were reported to be 90% and 11%, respectively (Lyon et al., 2010)

Urinary protein concentration dependes not only to pathologic states but also to the hour-to-hour or day-to-day urine dilution. Therefore, dipstick results should be interpreted together to USG in order to estimate the grade of proteinuria.

In a previous study (Zatelli et al, 2010) a scheme of interpretation of dipstick results in conjunction with USG values was suggested in dogs (Table 3) and a similar approach should be carried out in cats.

Table 3 Scheme of interpretation of dipstick in dogs. Samples with negative dipstick are likely non proteinuric (NP), samples with 2+ or more are likely proteinuric and samples with 1+ may or may not be proteinuric depending on the USG (modified by Zatelli et al, 2010). UPC: urinary protein-to-creatinine ratio; USG: urine specific gravity.

	Dipstick results		
	0+	1+	≥2+
USG ≤1012	NP	UPC necessary to confirm diagnosis	UPC necessary to grading
USG >1012 and <1030	NP	NP	UPC necessary to grading
USG ≥1030	NP	NP	UPC necessary to grading

Although the evaluation of protein excretion using the dipstick is considered a screening test, pre-analytical and analytical variables could affect clinical decisions. Alkaline urine, quaternary ammonium salts and chlorexidine could falsely increase protein results, and also the presence of abnormal pigments could interfere with the reagent pad color and reading (Stockham and Scott, 2008).

Some laboratories confirm the positivity of the dipstick with the sulphosalicylic acid (SSA) turbidometric test, especially in case of trace or 1+ results. SSA test is based on the denaturation of the proteins by the acid solution to form a precipitate that is detected as increased turbidity. It could be affected by false positivity due to some drugs and false negative due to alkaline urine. Compared to dipstick, it was shown to have higher specificity (25%) for the detection of albumins. (Lyon et al., 2010) Therefore, when applied as confirmation test after dipstick, SSA should be used in series instead of in parallel, in order to improve the specificity.

Dipstick and SSA allow an estimation of proteinuria and are not accurate for quantitative evaluation. Although the gold standard for an accurate quantification (and detection) of proteinuria is the measurements of proteins in a 24 hours urine collection (Monroe et al., 1989), in veterinary medicine this approach is impractical in clinical settings. Currently, the

quantification of proteinuria with the urinary protein-to-creatinine (UPC) ratio in spot urine sample is considered a reliable estimation of the daily protein excretion in cats (Adams et al., 1992).
Since creatinine is constantly filtered by glomerulus, the ratio of urinary protein with urinary creatinine allows the normalization with the hour-by-hour urinary dilution.

Concentration of urinary proteins is quantified by the means of automated biochemical analyzers in laboratories and different specific reagents are commercially available. Among these reagents, dye-binding methods are easy to use, relatively rapid and inexpensive. The most used are Pyrogallol red molybdate (PRM) and Coomassie brilliant blue (CBB). (Adams et al., 1992; Kuwahara et al., 2008; Williamset al., 2016; Fiorina et al., 2001; Martin et al., 2011). Other methods are based on protein precipitation and could be used as well (McDowell, 1985).
Urinary creatinine is measured with the same analytical methods used for serum creatinine (Stockham and Scott, 2008). However, in order to obtain accurate results, to measure urinary creatinine it is necessary to dilute urinary supernatants to low the concentration within the range of linearity of the method. In dogs it was demonstrated that the best dilution is 1:20 (Rossi et al., 2012).

IRIS staging based on proteinuria suggests 3 stages (Table 4) and the evaluation should be ideally done on the basis of at least two urine samples collected over a period of two weeks. Patients that are persistently borderline proteinuric should be re-evaluated within 2 months. IRIS and the ACVIM consensus statement recommends therapeutic intervention with antiproteinuric drugs when UPC ≥0.4 in cats with CKD (Lees 2005; Elliott and Watson, 2016).

Table 4 IRIS staging based on proteinuria

UPC value	Substage
<0.2	Non-proteinuric
0.2 – 0.4	Borderline proteinuric
>0.4	Proteinuric

Since the clinical decisions concerning proteinuria are currently based on quantification of urinary proteins with UPC ratio, it is of extreme importance to know and take into account all the possible source of biological and analytical variability, discussed below.

URINARY TRACT DISEASE. In the diagnostic approach to proteinuria in CKD it is mandatory to exclude pre-renal and post-renal causes of proteinuria. Urinary tract inflammation and hemorrhage are the main causes of post-renal increase of protein excretion (Grauer, 2011) and are possible concurrent finding in CKD in cats (Martinez-Ruzafa et al., 2012). In dogs UPC significantly increased only in case of macroscopic hematuria and was more likely to be high with concurrent hematuria and pyuria, according to a study (Vaden et al., 2004). Despite this, it is still recommended to role out post-renal inflammation before to interpret UPC values in CKD patients (Lees et al., 2005; Grauer, 2016). In cats, few information is available about the effect of hematuria and pyuria on UPC ratio but similar results are expected and similar approach is warranted. Interestingly, a recent study in cats highlighted mean UPC values of 0.70 ±0.19 in idiopathic cystitis yielding active urinary sediment (Panboon et al., 2017).

BIOLOGICAL VARIABILITY. In dogs, quantification of UPC in consecutive days is necessary when UPC is higher than 5, in order to determine a mean value representative of the actual value given a day-to-day variation of UPC (Nabity et al., 2007). However, in cats these values are rarely reached (Syme et al., 2006) and no information is available about this variability in cats. If necessary, it is possible to determine the mean value between consecutive samples on a pool of urine samples, in order to reduce the number of tests and the expense for the owner (LeVine et al., 2010). In dogs, a recent study demonstrated that intact male dogs could present persistent borderline proteinuria (UPC between 0.2 and 0.5) that after castration, tended to low at non-proteinuric range (UPC<0.2) (Bertieri et al., 2015). No information is available about the effect of neutering status on UPC in cats, but it is deemed that intact male could have higher urinary protein content, given the more abundant cauxin proteins in urine (Miyazaki et al., 2006). Moreover, urine collected at the clinical setting yielded UPC ratio higher compared to UPC measured in urine collected at home, suggesting an influence of stress on proteinuria (Duffy et al., 2015). However, it is not known whether also in cats the site of collection affect UPC. On the other hand it is known that in cats, UPC did not differ between samples

collected with cystocentesis and samples collected by manual compression of the bladder (Vihena et al., 2015).

ANALYTICAL VARIABILITY. In dogs it was shown that different pre-analytical and analytical procedures could affect UPC quantification, such as storage conditions, urine predilution to measure urinary creatinine and intra-assay variability (Rossi et al., 2012).
Another recent study in dogs showed biases between the two most common dye-binding methods for quantification of urinary proteins and one of two (PRM) the latter tended to underestimate protein concentration (Rossi et al., 2016). Also in cats there are reports demonstrating disagreements between analytical methods different to PRM and CBB. (Fernandes et al., 2005; Heeley, 2016). In dogs, disagreements was recently demonstrated between different labs also using the same reagent (PRM) but different biochemical analyzer and standard operative procedures (Rossi et al., 2015) and it is likely that this variability exist also for feline urinary proteins.
Hence, the UPC ratio can be affected by different assays principles and as a consequence cats with kidney diseases can be incorrectly sub-staged applying the IRIS guidelines.

Hypertension

Systolic blood pressure could be routinely assessed by indirect methods such as oscillometry and doppler ultrasonography. To obtain reliable values in the measurement of BP, it is important that a trained operator follow a standard protocol as described in a previous published guideline (Brown et al., 2007). Similar to any other analytical methods, the instrument should be validated for the cat and checked at leas annually (Brown et al., 2007). The patient should be acclimatized to the measurement condition in order to avoid the so called white-coat hypertension (WcHT). In animals WcHT is defined as the increase of SBP measured in clinical setting not detected at home or in general in case of stressful environment. Although the physiologic cause of the white-coat effect is not precisely known, it is deemed to be caused by the temporary sympatheic stimulation that could occur during the stressful clinical consultation (Zimmerman and Frohlich, 1990). In cats, this stress could occur during the transportation to the clinic and/or could occur due to the unfamiliar and relatively invasive procedures performed during the clinical visit (Belew et al., 1999). SBP could rise on average 30 mmHg above the actual value (both in healthy cats and in cats affected by CKD) and until 70 mmHg higher than baseline value (Belew et al., 1999). Hence WcHT is of concern in veterinary medicine since it interferes with the

SBP evaluation and a false diagnose of HT could occur. Moreover, this condition it's difficult to manage since the repeated measurement in clinical setting could reduce but not delete the WcHT. Although there is some evidence that the presence of white-coat hypertension in normotensive people is a risk factor for subsequent hypertensive damage, (Karter et al., 2003) there is no justification for treating white-coat hypertension in cats (Brown et al., 2007). Repeated measurements over time are therefore necessary to confirm the presence HT.

IRIS suggest a substaging of HT according to the degree of risk of target organ damage and whether there is evidences of target organ damage or complications (tables 5).

Table 5 IRIS sub-staging according to systolic blood pressure (SBP)

SBP (mmHg)	Sub-stage	Acronym	Risk of future target organ damage
<150	Normotensive	AP0	Minimal
150-159	Borderline hypertensive	AP1	Low
160-179	Hypertensive	AP2	Moderate
>180	Severely hypertensive	AP3	High

General aims

The first general aim of this thesis was the preliminary evaluation of pre-analytical and analytical variability of some laboratory parameters used in International Interest Society (IRIS) staging of feline chronic kidney disease (CKD). Specifically, to evaluate:

- whether the pre-analytical and analytical variability of urinary protein-to-creatinine (UPC) ratio (such as storage conditions, effect of creatinine predilution, and imprecision and method-dependent difference) could affect proteinuria interpretation in clinical practice (study 1);
- whether the presence of hematuria or pyuria affects the urinary protein concentration and UPC ratio in feline urine (study 2);
- whether the serum concentration of symmetric dimethylarginine (SDMA) in Birman cats, which have higher physiological serum creatinine, could be a better biomarker to estimate theglomerular filtration rate (study 3)

Then, this thesis was aimed to assess the usefulness of novel biomarkers of hypertension and proteinuria in cats with CKD in order to define the pattern typical of each IRIS stage and the role of these biomarker as prognostic factors (study 4). Specifically, serum biomarkers that in other species increase in hypertension and CKD, such as big endothelin-1 (study 4.1) homocysteine (study 4.2), aldosterone (study 4.3) or biomarkers such as big endothelin-1 (study 4.1), alpha-1 microglobulin (study 4.4) and tubular proteins (study 4.5) increase also in cats with CKD and may therefore work as early biomarkers for diagnosis or prognosis of CKD or as ancillary tests to preliminary select cats potentially affected by hypertension, avoiding procedures to measure blood pressure in normotensive cats. Our hypothesis is that both serum biomarkers increase and urinary protein patterns change in cats with CKD, as happens in humans and dogs.

Description of the studies

1. Evaluation of pre-analytic and analytic variability of urine protein-to-creatinine ratio in cats

Introduction and aims

The quantification of proteinuria with the urinary protein-to-creatinine (UPC) ratio in spot urine sample is considered a reliable estimation of the daily protein excretion in cats (Monroe er al., 1989; Adams et al., 1992) and it's mandatory in diagnostic approach of CKD and staging according to the International Interest Renal Society (IRIS) guidelines (Elliot and Watson, 2016).

In human medicine it was shown that different methods for urinary protein quantification yielded discordant results (Chambers et al., 1989; Dube et al., 2005) and efforts were made to improve agreement (Wimsatt and Lott, 1987; Marshall and Williams, 2000). Similarly, in dogs, the UPC ratio can be affected by different assays principles and as a consequence dogs with kidney diseases can be incorrectly sub-staged applying the IRIS guidelines. A recent study in dogs showed biases between CBB and PRM in quantification of urinary protein in canine urine and the latter tended to underestimate protein concentration (Rossi et al., 2016). Moreover, also in cats there are reports demonstrating disagreements between analytical methods different to PRM and CBB (Fernandes et al., 2005; Heeley, 2016). On this regard, it's important to highlight that the IRIS guidelines do not specify which method should be used to assess the thresholds proposed in sub-staging feline patients with CKD.

Other factors, such as different pre-analytical procedure in different laboratories, storage of supernatants or pre-dilution for urinary creatinine quantification have been shown to influence the quantification of urinary protein in dogs (Rossi et al., 2012; Rossi et al., 2015).

To our knowledge there is no information available about pre-analytical and analytical factors that may affect the measurement of proteinuria.

Therefore, the aims of this study were to determine whether preanalytic and analytic factors affect the evaluation of the UPC ratio in cats. Specifically, the influence of preanalytic dilution for creatinine quantification, the intra-assay repeatability of UPC ratio measurement and the effect of storage at different temperatures and times were evaluated. In addition, agreement between two dye-binding methods (PRM and CBB methods) for measurement of total protein in feline urine was determined.

Material and methods

Animals and sample collection

One hundred seventy-four urine samples were prospectively collected from client-owned cats presented for routine diagnostic investigations

Samples were collected from January 2015 to February 2016 at the Internal Medicine Unit of the Department of Veterinary Medicine (University of Milan) and at a private clinical practice in Milan (Ospedale Veterinario Città di Pavia) during routine health screen, under informed consent signed by the owners. According to the ethical committee statements of the University of Milan (number 2/2016), biological samples collected in this setting could be used also for research purposes.

Due to the analytical nature of this study, cats were enrolled irrespective of age, sex and breed or underlining disease and also cats with diseases that could affect urine composition (e.g. CKD, lower urinary tract inflammation, neoplasia, etc.) were included.

Eight to 10 mL of urine samples were collected from each cat by ultrasonographically-guided cystocentesis. Samples were sent within the syringe to the respective internal clinical pathology laboratories (labelled as “Lab 1” for university of Milan and “Lab 2” for Ospedale Veterinario “Città di Pavia”).

Urinalysis

Five millilitres of urine were transferred from the syringe to a sterile conical tube and were macroscopically evaluated for physical properties (color and turbidity) and assayed with dipstick for a semi-quantitative chemical analysis (Combur 10 test, Roche diagnostics, Risch-Rotkreuz, Switzerland). Urine specific gravity (USG) was determined by a handheld refractometerd calibrated daily with distilled water (Clinical Refractometer, model 105, Sper Scientific, Scottsdale, AZ, USA).

In order to perform sediment evaluation and supernatant collection, tubes were centrifuged at 1250 rpm for 5 minutes (450G) (Hermle Z300, Labnet international, Edison, NJ, USA). Then, 4.75 mL of supernatant was removed and transferred in other tubes for subsequent diagnostic biochemical analysis and for study purposes (see below). Supernatants were removed by suction using a dispensable pipette according to current guidelines (ECLM, 2000) in order to avoid loss of sediment and supernatant contamination by elements of the sediment.

Sediments were resuspended in the remaining 0,25 mL supernatant and 50 µL were placed on a slide, covered with a microscope coverslip (24x32 mm) and evaluated microscopically al 100x (low power field, LPF) and 400x (high power field, HPF) magnifications.

Bacteriuria, spermaturia, lipiduria, and the presence of epithelial cells, casts, and crystals were recorded according to a semiquantitative scale (rare, moderate, abundant, or very abundant) whereas erythrocytes and leukocytes were quantified as the mean on 10 HPF. Sediments were classified as active on the basis of the presence of one or more of the following findings: >5 RBCs /HPF, >5 WBCs /HPF or both. Samples without any of these listed findings were classified as inactive.

Supernatants enrolled in “Lab 1” were used fresh for the analytical procedures described below. Supernatants collected at “Lab 2” were aliquoted (approximately 2 mL each sample) and stored at −20° within 2 hours from collection. Then, aliquots were shipped in batch under controlled temperature to “Lab 1” for inclusion in method comparison study (see below).

Analytical methods

Two commercially available colorimetric test kits were used for protein quantification on urine supernatants in “Lab 1”, one based on PRM (Urine proteins, Sentinel diagnostics, Milan, Italy) and the other based on CBB (Total protein coomassie urine, Far Diagnostics, Pescantina (VR), Italy). The concentration of urinary protein was expressed in mg/dL either for PRM (UP^{PRM}) or for CBB (UP^{CBB}). Both methods were performed according to manufacturer’s instructions and were calibrated with the standards provided by the manufacturers. Specifically, PRM standard was stated to be “urinary protein” with no specification of the particular nature of the protein content whereas CBB standard was bovine serum albumin. The protein concentration of the PRM standards provided with the different lots used during the study period ranged from 109 to 122 mg/dL whereas the concentration of CBB standards was 100 mg/dL in all the lots used.

Preliminary assays run in our lab demonstrated that PRM method was linear up to 210 mg/dL as reported by the manufacturer whereas CBB method, independently on the limit of linearity indicated by the producer, loses linearity at concentration higher than 120 mg/dL. Therefore, when CBB yielded values higher than 120 mg/dL, supernatants were diluted 1:5 with distilled water; then, samples were re-run with both PRM and CBB and the actual values were calculated based on the dilution factor.

Urinary creatinine concentration (UC) was measured with the modified Jaffè method (Creatinina, Real-Time Diagnostics, Viterbo, Italy) and was expressed as mg/dL.

When CBB method was applied in a session of work, PRM and Jaffe methods were run first, due to the peculiarity of CBB reagent to stain the reagent needle of the automated analyser and the theoretical possibility of contamination and interference of Coomassie dye in the subsequent reaction. Then, an automated cleaning of the needle at the end of the session was performed.

Because urinary creatinine concentration frequently exceeds the range of linearity of the method, supernatants were diluted 1:20 with distilled water in order to measure urinary creatinine and then the actual values were calculated.

Except when differently specified, biochemical tests were performed in triplicate and the mean values were used for data analysis.

All tests were performed with an automated biochemical analyser in Lab1 (Cobas Mira, Roche Diagnostics, Basel, Switzerland) and all methods were daily controlled with a solution known protein concentration (70.6 mg/dL, level 3 calibration solution, Siemens Dimension® UCFP calibrator, Muenchen, Germany) and calibrated if necessary.

UPC ratios obtained with PRM (UPC^{PRM}) and, UPC ratios obtained with CBB (UPC^{CBB}) were calculated for each method.

Experimental procedures

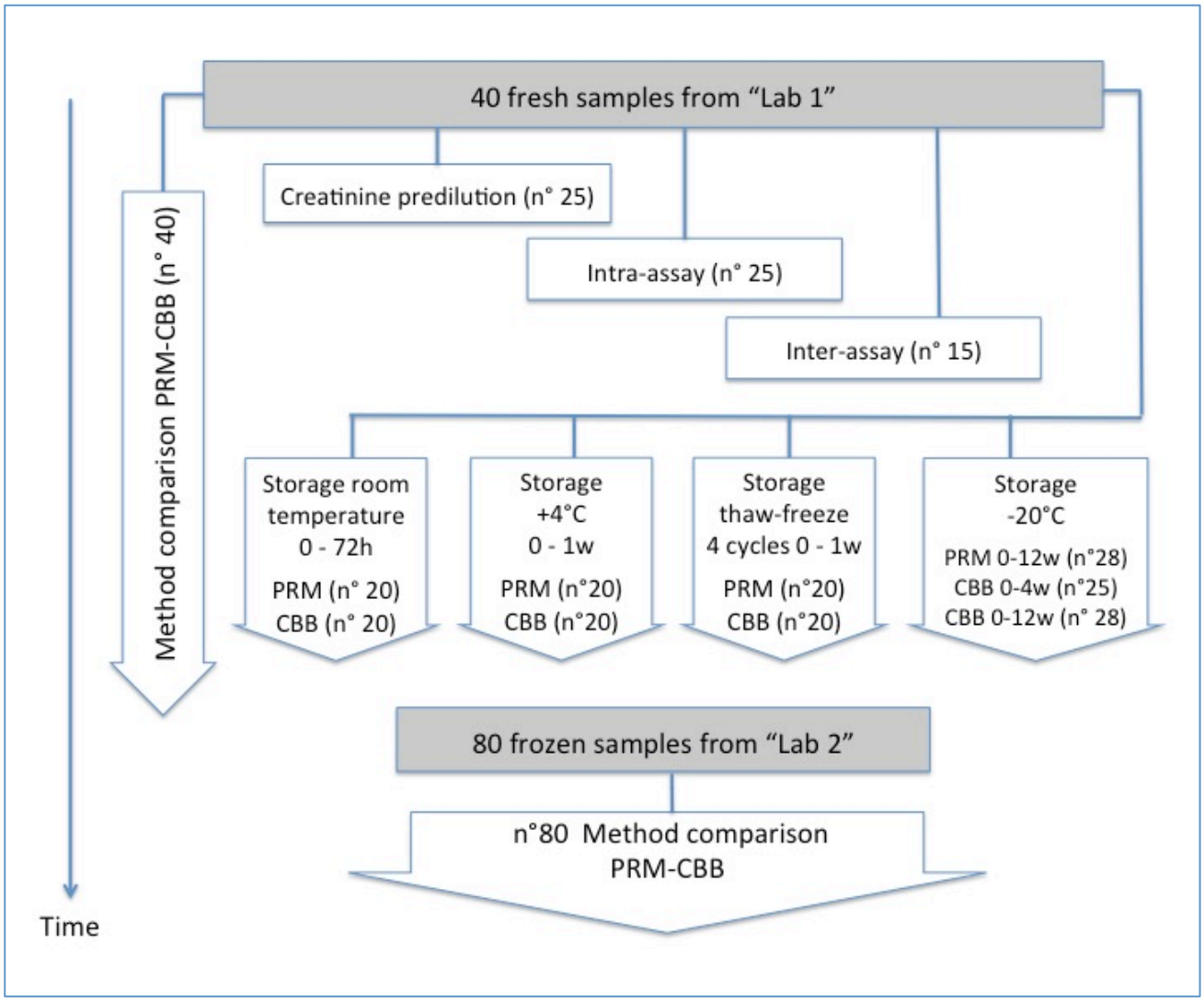
The flow of the study design with the different analysis performed and described below is shown in Figure 1.

Effect of predilution of urine samples— Three aliquots of 25 fresh urine supernatants were diluted 1:10, 1:20, and 1:100 and assayed for creatinine concentration. Protein concentration was also determined with PRM, and the UPC ratio corresponding to each creatinine dilution was calculated.

Intra-assay repeatability— The intra-assay imprecision was assessed on twenty fresh urine supernatants, testing samples 20 consecutive times in the same run for protein concentration (with both PRM and CBB methods) and for creatinine concentration and the UPC ratio was calculated. Mean, SD and CV (calculated as CV = SD/mean X 100) for UP^{PRM}, UP^{CBB}, UC and thus UPC ratio for each method were calculated first on the whole

set of samples and then considering separately the results from samples with active and inactive sediment.

Figure 1 Flow chart of this study. A preliminary evaluation of the best supernatant dilution for creatinine measurement was performed and intra-assay and inter-assay variability was evaluated. Then, the effect of different storage conditions was assessed. In the meanwhile, method comparison study on the fresh urine samples included in those evaluations was carried out. After results of storage at -20°C, samples form Lab2 were included in method comparison study



Inter-assay repeatability— The inter-assay imprecision was assessed in 15 samples, immediately aliquoted after sampling and stored at -20°C. Each sample was measured on 5 consecutive working days. Urine proteins were measured with both methods (PRM and CBB), urine creatinine was also measured to calculate the UPC ratio with each method. Mean, SD and CV (calculated as CV = SD/mean X 100) were calculated for UP^{PRM}, UP^{CBB}, UC and thus UPC ratio for each method.

Effect of storage duration and temperature— Fresh urinary supernatants were included in this evaluation. To assess short-term stability at room temperature (20°C), 40 samples (20 using PRM and 20 using CBB) were assayed within 2 hours from collection (T_0) and aliquoted in 3 tubes (300 µL); aliquots were left in closed tubes on the working desk of the lab and they were assayed at 2, 4 and 6 hours from T_0;

To assess the effect of refrigeration, 40 samples (20 using PRM and 20 using CBB) were assayed at T_0, then seven aliquots (300µL) were stored at 4°C and analyzed 24, 48, and 72 hours and 1 week after storage.

To assess the effect of repeated thawing-freezing cycles was evaluated in 40 samples (20 using PRM and 20 using CBB): 1 aliquot of 1 mL was stored at -20°C and was thawed, analysed and frozen again at 24, 48 and 72 hours and 1 week after collection (4 thaw-freeze cycles).

To assess stability at freezing temperature, 28 samples were tested at T_0, then 6 aliquots (300 µL) were stored at –20°C and analysed 1, 2, 3, 4, 8 and 12 weeks after storage, using PRM for protein quantification at each checkpoint. Twenty-five supernatants were assayed at T_0 for UP^{CBB} and UC, were immediately stored in one aliquot (300 µL) at −20°C and evaluated again at 1 month. Stability at 3 months were evaluated on further 28 samples (different from those included in the previous evaluation) at T_0 and 3 months for UP^{CBB} and UC.

In all the tests previously described, refrigerated or frozen samples were allowed to gently warm or thaw at room temperature and subsequently vortexed.

Method comparison study— Forty samples from “Lab1” and 80 samples from “Lab2” were included. Supernatants sent to “Lab1” were analysed fresh within 3 hours from collection, while supernatants from “Lab2” have been stored no longer than 4 weeks at -20°C before the assay.

Urine protein concentration was measured using both PRM and CBB methods, creatinine concentration was measured to allow the calculation of UPC ratios for each method.

UPC ratios obtained with both methods (PRM and CBB) were classified as NP, BP or P according to the IRIS staging system.

Statistical Analysis

A commercially available software (MedCalc® Statistical Software, version 16.8.4, Ostend, Belgium) was used. A P value <0.05 was considered statistically significant. Normality of variables was assessed by Kolmogorov-Smirnov test.

For the evaluation focused on the effect of predilution of urine supernatants, creatinine concentrations, measured by use of the three dilutions and the corresponding UPC ratios, were compared with Friedman test, followed by Wilcoxon signed rank test to compare the results between single dilutions; the agreement between results was tested with Bland-Altman and Passing-Bablok tests; concordance in classify sample according to IRIS guidelines for proteinuria (NP, BP, P) in cats was tested with Cohen k test (see below for further details).

For the evaluation focused on the intra-assay variability, the possible correlation between intra-assay CV of urinary protein concentration, urinary creatinine concentration or UPC ratio, and the actual values of each of these variables, was investigated with Spearman correlation test. Mann-Whitney *U* test was applied to investigate difference in UP, UC and UPC ratios between samples with active and inactive sediment.

For the evaluation focused on the influence of different storage conditions on UP, UC and UPC ratios, results obtained at different checkpoint were analyzed using Friedman test followed by multiple comparisons using the Wilcoxon signed rank test adjusted with the Bonferroni correction in case of >2 checkpoint (i.e. room temperature, 4°C, repeated freeze-thaw cycles and –20°C using PRM). Wilcoxon signed rank test was also used for samples stored at –20°C and assayed with CBB. These tests were performed on the whole set of samples and on the data grouped according to the presence or absence of an active sediment. In order to assess the clinical relevance of the variation due to the storage conditions, at each checkpoint, UPC results were considered clinically different whenever the mean value was outside the range determined applying the inter-assay CV to the respective mean T_0 values. To this aim, the inter-assay CV values found in this study were used (see Results).

For the method comparison study, the UP values obtained with PRM and CBB were compared to each other with Wilcoxon signed rank test to assess difference and assayed for correlation with the Spearman test. The same analysis has been run to compare the UPC ratios calculated using the PRM and the CBB method. The agreement between the two methods was assessed by Passing-Bablok and Bland–Altman tests.

The concordance of the two methods in classifying samples according to IRIS staging of proteinuria was assayed with the Cohen's kappa (k) concordance test. The Cohen's k coefficient was used to define concordance as "very good" (k = 0.8–1), "good" (k = 0.6–0.8), moderate (k = 0.4–0.6), "fair" (k = 0.2–0.4), "poor" (k = 0.0–0.2) or "absent" (k <0) (Landis and Koch, 1977). Method comparison study tests were performed for the whole set of data and for the sub-sets of samples grouped according to the presence or absence of active sediment.

Results

Effect of predilution of urine samples

Median of UC concentration measured at 1:10, 1:20 and 1:100 dilutions were 191.4 mg/dL (mean ± SD: 225.2 ± 117.0 mg/dL), 188.1 mg/dL (mean ± SD: 227.2 ± 126.4 mg/dL) and 180.3 mg/dL (mean ± SD: 219.1 ± 123.8 mg/dL) respectively.

Median UPC ratios measured at 1:10, 1:20 and 1:100 dilutions were 0.16 (mean ± SD: 0.23 ± 0.24), 0.17 (mean ± SD: 0.24 ± 0.24) and 0.16 (mean ± SD: 0.24 ± 0.26), respectively. No significant differences were found between the three dilutions for both UC (P = 0,468) and UPC (P = 0.540).

Comparing dilutions with Bland-Altman and Passing-Bablok tests, only a constant error between UPC calculated at 1:20 dilution and UPC calculated at 1:100 dilution was found (Figure 2 and 3).

Agreement in classifying samples according to IRIS stages was defined "very good" in all the three comparisons (1:20 vs 1:10, k = 0.83; 1:20 vs 1:100, k = 1; 1:10 vs 1:100, k = 0.83). Only two samples yielded different IRIS stage when measured using 1:10 compared to other dilutions (one NP instead of BP and one BP instead of NP).

Intra- and inter-assay variability

Descriptive statistics of the samples included in intra-assay and inter-assay evaluation and the respective CVs with regard of UP^{PRM}, UP^{CBB}, UC, UPC^{PRM} and UPC^{CBB} are shown in Table X. Test for normality revealed a non-Gaussian distribution for both UP, UC and thus for UPC.

The CV was lower for the UC than for UP (and UPC ratio) measured with both PRM and CBB. CBB method appeared more precise than the PRM method. The effect of this variability on sub-staging of sample according to IRIS guidelines was assessed on 4 urine

samples that had UPC ratios close to the threshold values (i.e. 0.2 and 0.4) and is shown in Table 6.

No significant differences were found between mean values of UP^{PRM}, UP^{CBB}, UC, UPC^{PRM} and UPC^{CBB} between samples with active and inactive sediment.

No significant correlations were found comparing intra-assay CV and mean values of UP^{PRM} (r = –0.08; P = 0.72), UP^{CBB} (r = –0.29; P = 0.220), UC (r = –0.01; P = 0.95), UPC^{PRM} (r = –0.23; P = 0.33) and UPC^{CBB} (r = –0.19; P = 0.42).

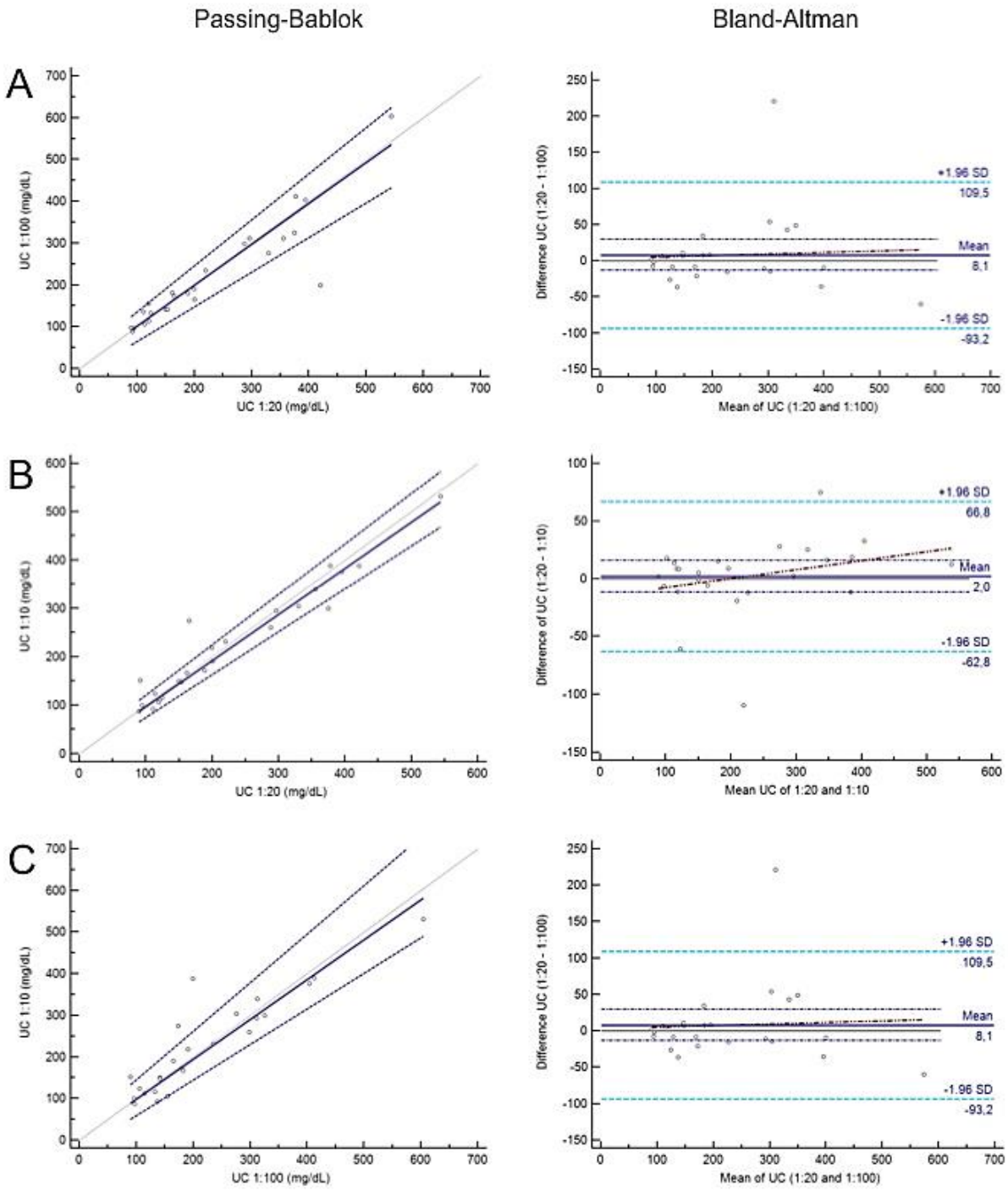


Figure 2 Passing Bablok (left) and Bland-Altman (right) plots regarding the comparison of urinary creatinine (UC) concentration between dilutions 1:20 vs. 1:100 (A), 1:20 vs. 1:10 (B) and 1:100 vs. 1:10 (C), obtained from 25 samples. In Passing Bablok plots, the blue line is the correlation, the gray line shows best fit and the blue dotted lines 95% CI. In Bland–Altman plots, X axes represent the average between the two dilutions, and the Y axes the indicate the difference between dilutions; the grey line shows the zero bias, the blue solid with the dashed blue lines represent the bias and 95% confidence interval (CI), respectively; the light blue dashed lines are the limits of agreement and the red dotted line is the regression line.

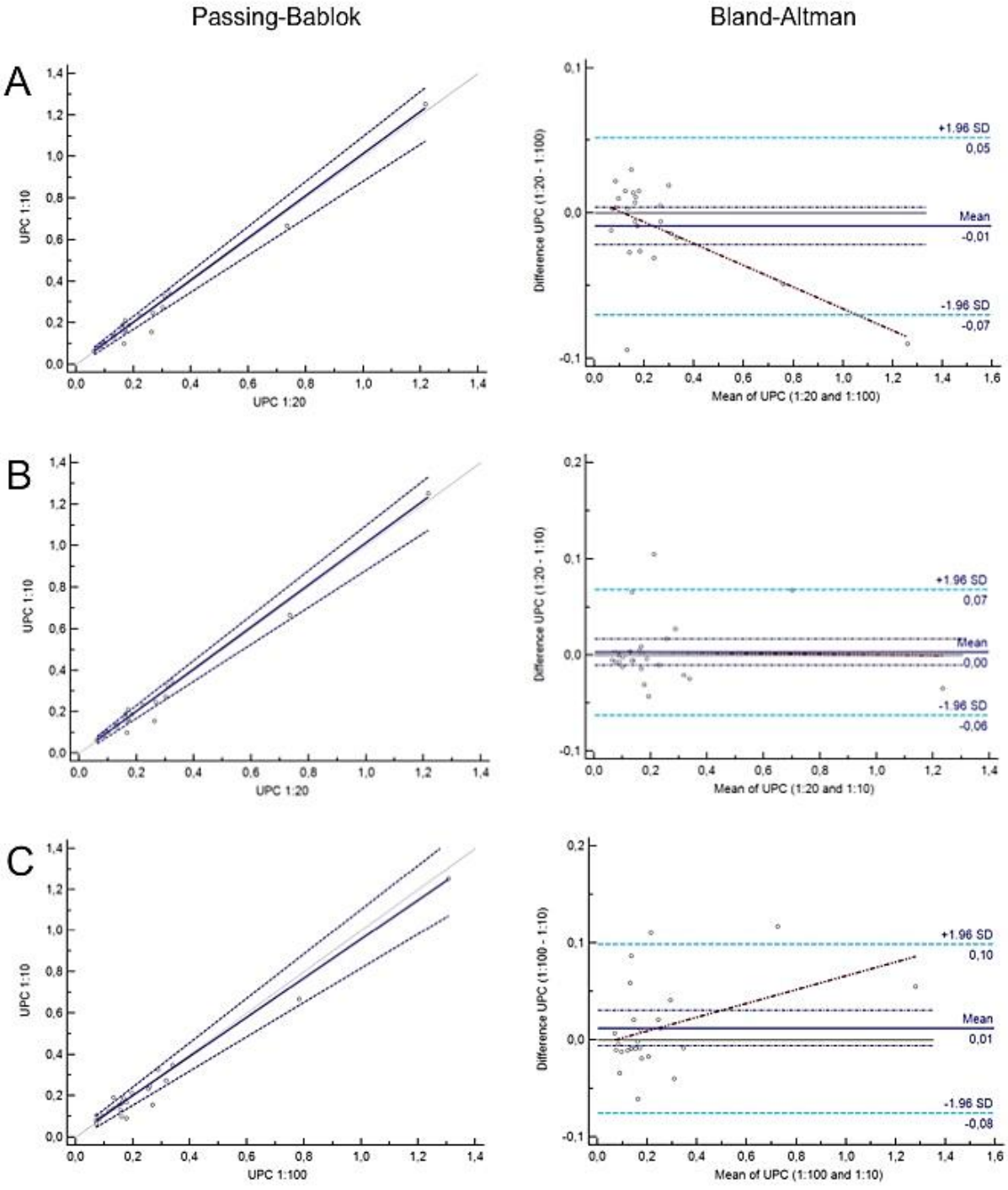


Figure 3 Passing Bablok (left) and Bland-Altman (right) plots regarding the comparison of urinary protein-to-creatinine (UPC) ratio between dilutions 1:20 vs. 1:100 (A), 1:20 vs. 1:10 (B) and 1:100 vs. 1:10 (C), obtained from 25 samples. In Passing Bablok plots, the blue line is the correlation, the gray line shows best fit and the blue dotted lines 95% CI. In Bland–Altman plots, X axes represent the average between the two dilutions, and the Y axes the indicate the difference between dilutions; the grey line shows the zero bias, the blue solid with the dashed blue lines represent the bias and 95% confidence interval (CI), respectively; the light blue dashed lines are the limits of agreement and the red dotted line is the regression line.

Table 1 Precision tests of protein concentration measured with PRM and CBB, creatinine concentration and urinary protein-to-creatinine ratio calculated with both methods. Intra-assay repeatability was evaluated in 20 samples (11 with active and 9 with inactive sediment) tested 20 consecutive times. Inter-assay repeatability was evaluated in 15 samples tested in 5 consecutive days. UP, UC and UPC values are described as median and range in brackets; CV values are described as mean ± SD.

	UP^{PRM}		UP^{CBB}		UC		UPC^{PRM}		UPC^{CBB}	
	UP concentration (mg/dL)	CV (%)	UP concentration (mg/dL)	CV (%)	UC concentration (mg/dL)	CV (%)	UPC ratio	CV (%)	UPC ratio	CV (%)
Intra-assay all samples	61.6 (22.8-858.6)	8.4 ±5.2	87.2 (33.4-614.8)	5.6 ±2.6	152.9 (35.3-517.7)	3.4 ±2.5	0.32 (0.05-24.32)	9.5 ±4.8	0.62 (0.15-17.41)	7.2 ±2.6
Intra-assay active sediment	56.5 (22.8-455.6)	9.3 ±6.8	82.8 (43.4-595.0)	5.5 ±2.1	152.8 (70.0-468.5)	3.7 ±2.8	0.32 (0.04-6.6)	10.4 ±6.4	0.61 (0.16-7.06)	7.1 ±2.5
Intra-assay inactive sediment	45.1 (23,9-78.1)	7.9 ±0.8	57.5 (33.4-101.7)	7.3 ±1.4	184.6 (93.9-374.4)	3.8 ±2.4	0.19 (0.16-0.27)	8.2 ±1.1	0.29 (0.15-0.41)	8.3 ±1.9
Inter-assay all samples	27.4 (9.8-518.4)	10.8 ±3.2	51.6 (18.2-314.6)	10.9 ±4.5	176.3 (59.3-426.8)	6.6 ±2.7	0.15 (0.06-6.59)	16.4 ±9.4	0.26 (0.07-3.98)	17.8 ±3.1

UP, urinary protein; UP^{PRM}, urinary protein measured with pyrogallol red-molybdate; UP^{CBB}, urinary protein measured with Coomassie brilliant blue; UC, urinary creatinine, UPC, urinary protein-to-creatinine ratio; UPC^{PRM} urinary protein-to-creatinine ratio measured with pyrogallol red-molybdate; UPC^{CBB} urinary protein-to-creatinine ratio measured with coomassie brilliant blue

Table 7 Frequency of misclassification of 4 feline urines with UPC ratios close to IRIS thresholds. When tested with PRM, 2 samples yielded UPC values close to the two IRIS cut-off (0.2 and 0.4). Similarly, two other different samples yielded UPC values close to the same two cut-off when tested with CBB. Number (and percentage) of shifts of IRIS stage out of the 20 repeated measurements in these samples were countered.

		UPC same stage	UPC different stage
UPC^{PRM}	BP (UPC =0.22)	17 (85%)	3 (15%) NP
	P (UPC =0.42)	13 (65%)	7 (35%) BP
UPC^{CBB}	BP (UPC =0.22)	18 (90%)	2 (10%) NP
	P (UPC =0.41)	11 (55%)	9 (45%) BP

UPC, urinary protein-to-creatinine ratio; UPC^{PRM} urinary protein-to-creatinine ratio measured with pyrogallol red-molybdate; UPC^{CBB} urinary protein-to-creatinine ratio measured with coomassie brilliant blue; BP, borderline proteinuric; P, proteinuric

Storage

No significant differences were found for UP, UC and UPC using both PRM and CBB when stored at room temperature for 6 hours, at +4°C for 1 week and after four thaw-freeze cycles within 1 week (Figure 4). When stored at -20°C (Figure 5), UP^{PRM} did not change during 12 weeks of storage in the whole set of samples and in the inactive sediment, whereas in active sediment set of samples was significant different (P = 0.002) highlighting higher values at 3rd week compared to T_0, 4th and 12th weeks according to the post-hoc analysis. UC significantly differed in the whole caseload (P = 0.047) but no differences were found in the active and inactive subsets. UPC^{PRM} significantly differed in the whole caseload (lower values at 8th week compared to T_0, 1st and 2nd weeks and lower values at 12th compared to 2nd week), and in the sets of active (higher values at 3rd week compared to T_0, 4th, 8th and 12th weeks) and inactive sediment (lower values at 8th week compared to T_0 and 2nd weeks). When storage stability at -20°C was tested with CBB (Figure 5), at 4 weeks UP^{CBB} and UPC^{CBB} did not significantly differed in any of the sets of samples. At 12 weeks (Figure 5) mean UP^{CBB} significantly differed (P = 0.046) in the whole set of samples, highlighting lower values at 12th week, whereas active and inactive sets did not. Conversely UPC^{CBB} did not significantly change in any of the sets of samples. All the previous UPC mean values at any checkpoint (including those significantly different) were within the range determined by the inter-assay variability applied to the respective UPC mean value at T_0 showing no clinical relevance of the statistical variation.

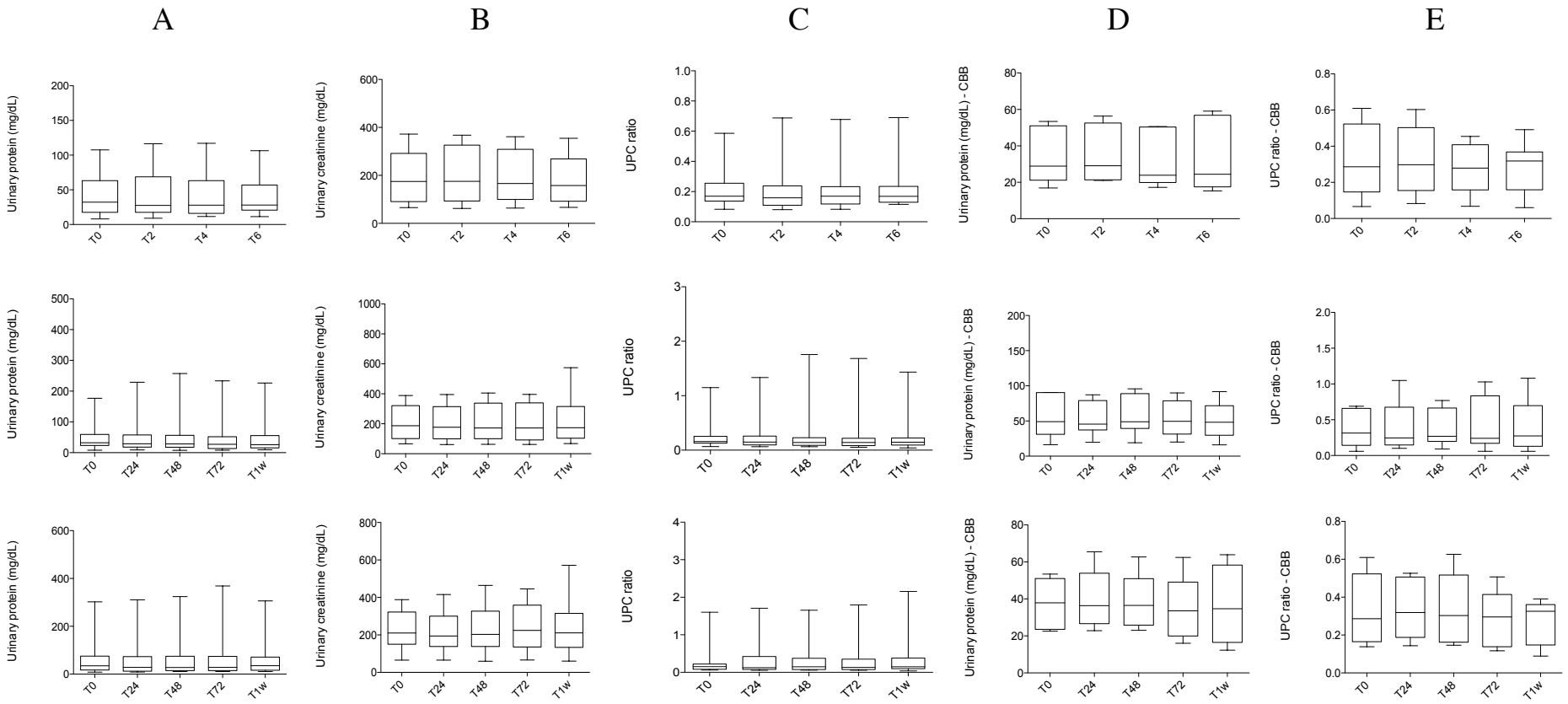


Figure 1 Effect of storage at room temperature within 6 hours (upper row), at +4°C for 1 week (middle row) and at thaw-freeze cycles for four cycles (lower row) for UP^{PRM} (A), UPC^{PRM}(B), UC (C), UP^{CBB} (D) and UPC^{CBB}(E). Box plots represent the interquartile range (IQR), and the horizontal line represents the median value of each group. Whiskers extend to the furthest observations within ± 1.5 IQRs of the first or third quartile

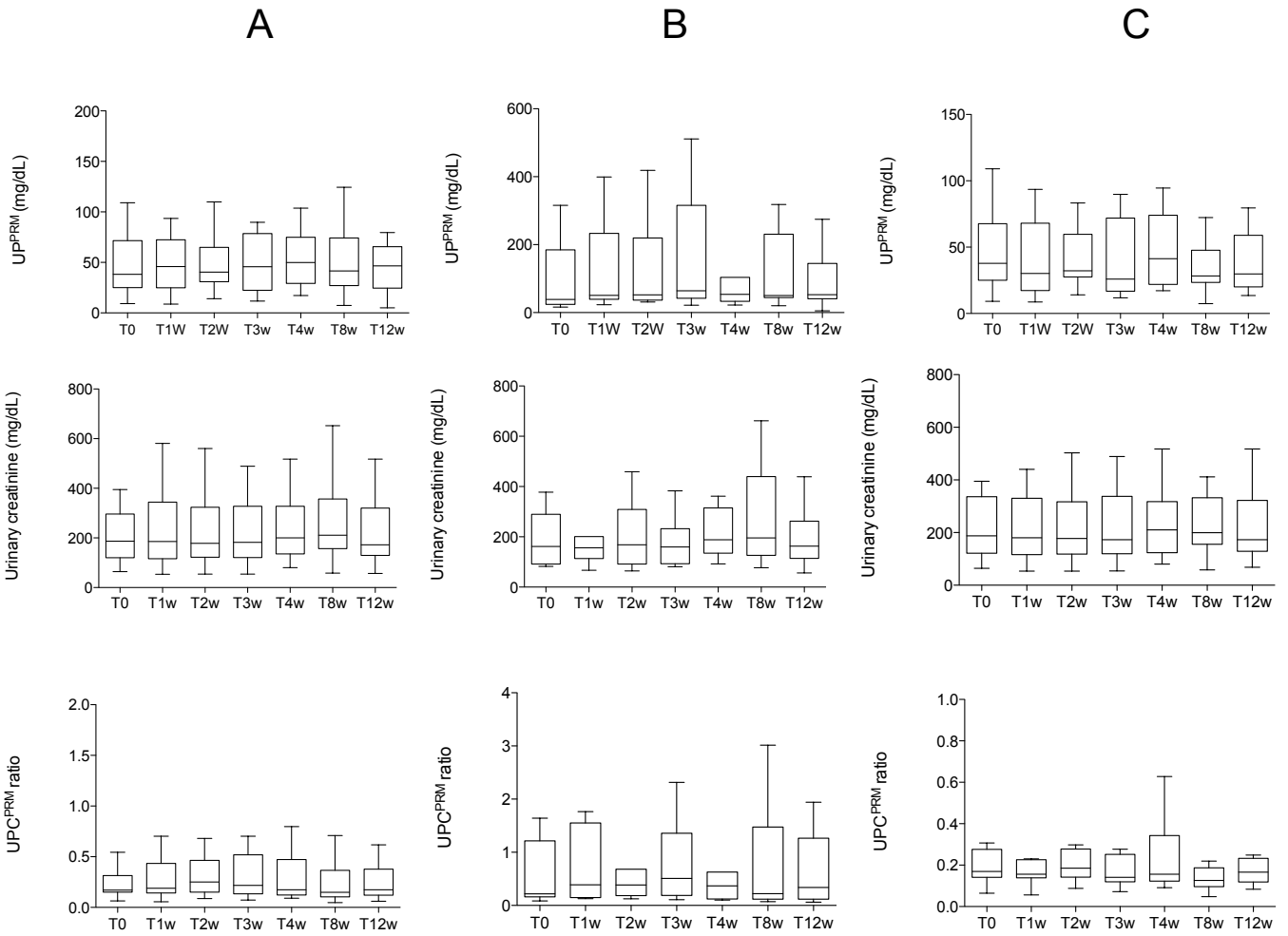


Figure 5 Effect of storage at -20°C for UP^{PRM} (upper row), urinary creatinine (middle row) and UPC^{PRM} (lower row). A: All samples (n = 28). B: Samples with active sediment (n = 11). C: Samples with inactive sediment (n = 17). Box plots represent the interquartile range (IQR), and the horizontal line represents the median value of each group. Whiskers extend to the furthest observations within ± 1.5 IQRs of the first or third quartile.

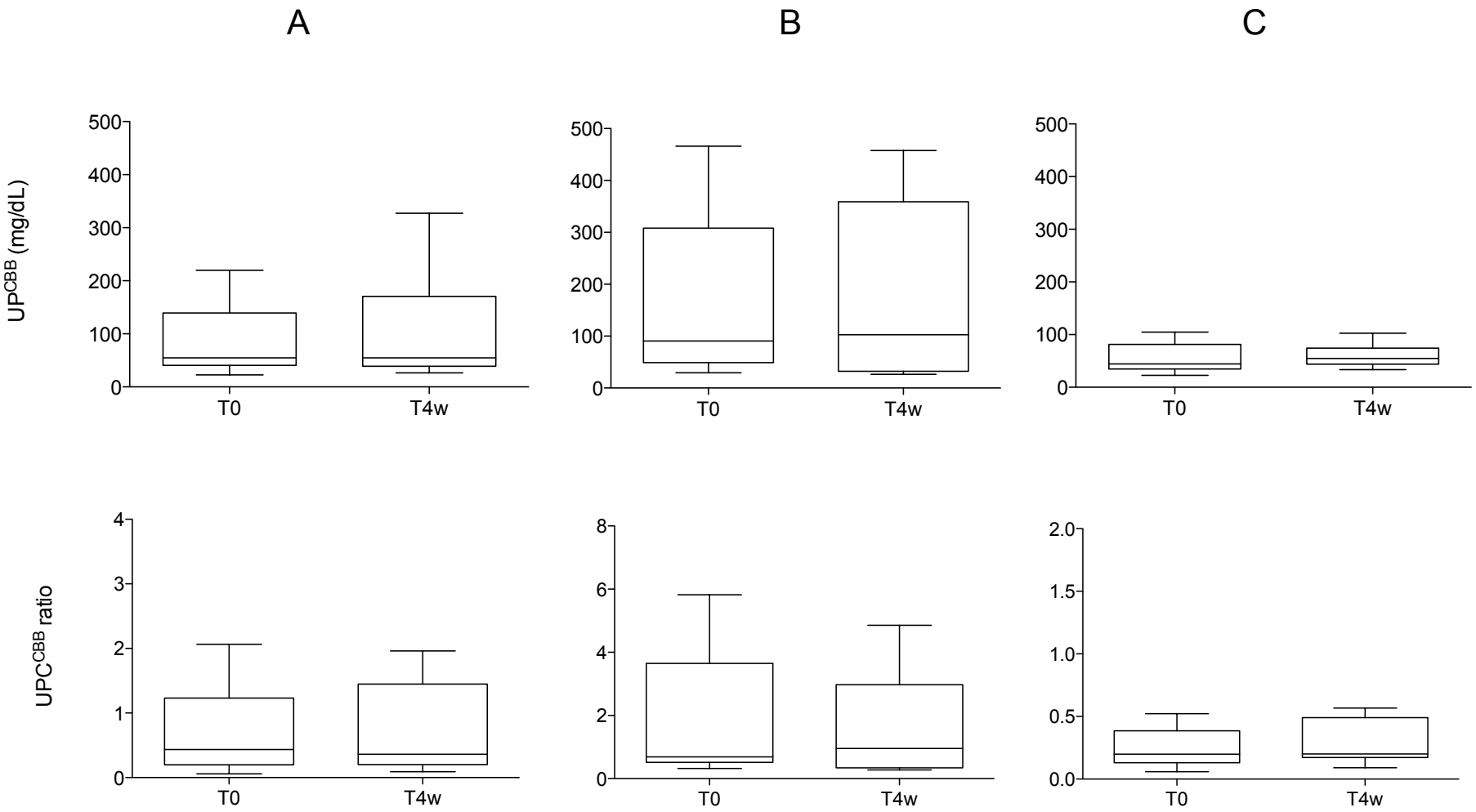


Figure 6 Effect of storage at -20°C for UP^{CBB} (upper row) and UPC^{CBB} (bottom row) after 4 weeks (T4w). A— All samples (n = 25). B— Samples with active sediment (n = 11). C— Samples with inactive sediment (n = 14). Box plots represent the interquartile range (IQR), and the horizontal line represents the median value of each group. Whiskers extend to the furthest observations within ± 1.5 IQRs of the first or third quartile

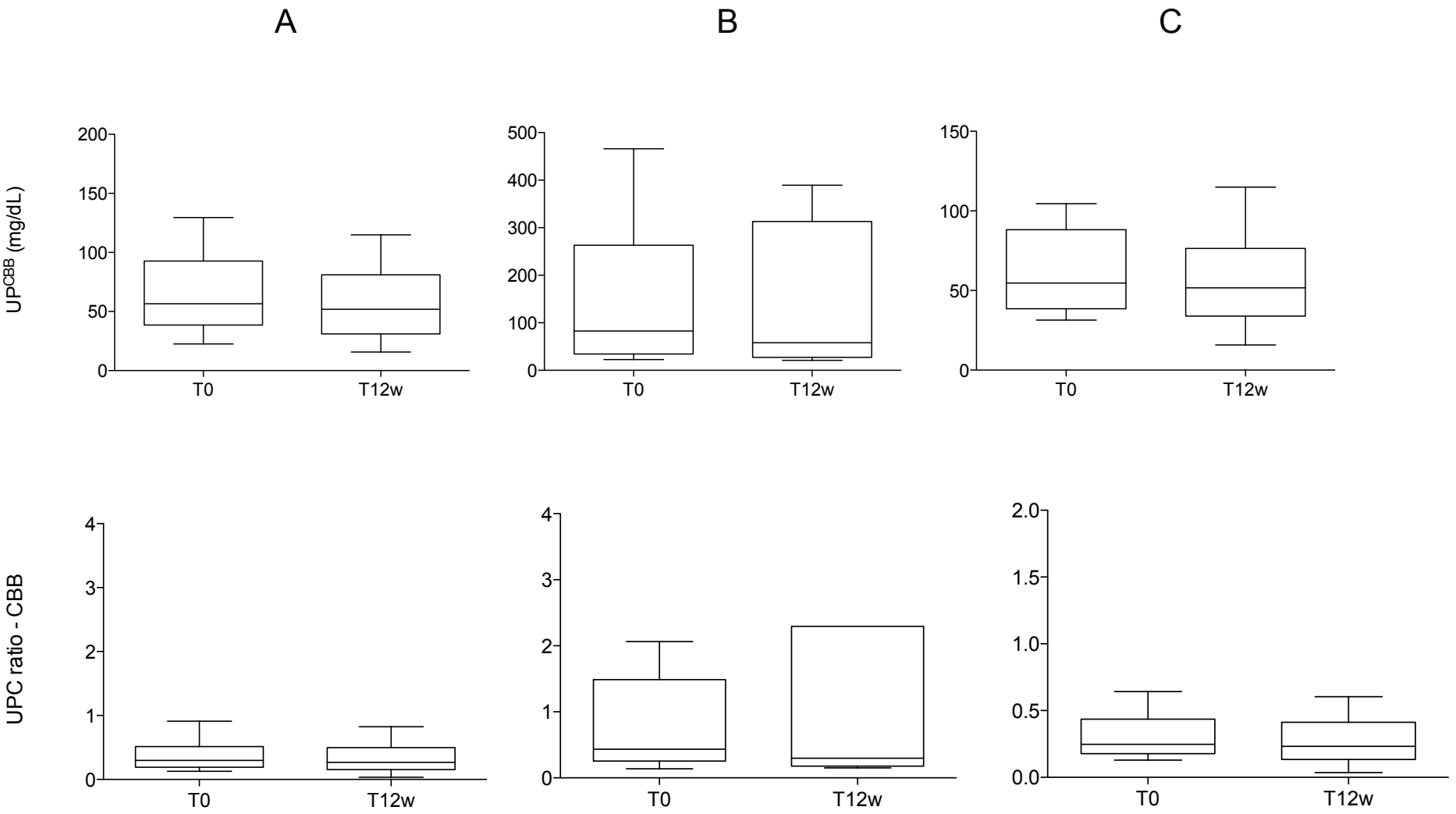


Figure 7 Effect of storage at -20°C for UP^{CBB} (upper row) and UPC^{CBB} (bottom row) after 12 weeks. A— All samples (n = 28). B— Samples with active sediment (n = 13). C— Samples with inactive sediment (n = 15). Box plots represent the interquartile range (IQR), and the horizontal line represents the median value of each group. Whiskers extend to the furthest observations within ± 1.5 IQRs of the first or third quartile.

Method comparison study

Data referred to the whole caseload or to samples with inactive or active sediment are reported in Table 7.

Forty-one (38.7%) urinary samples had an active sediment, while 65 (61.3%) had an inactive sediment. The most common sediment alteration was hematuria (68.3%), followed by leukocyturia (24.4%) and hematuria and leukocyturia (7.3%).

Using PRM, 66, 17 and 37 samples were classified as N, BP and P, respectively, whereas using CBB were 45, 25 and 50, respectively.

CBB yielded constantly higher UP and UPC ratios compared to PRM and the difference was significant ($P < 0.0001$) in all sets of samples.

Urinary protein (PRM: $P = 0.0146$, CBB: $P = 0.0104$) and UPC ratio (PRM: $P = 0.0035$, CBB: $P = 0.0087$) were significantly different between samples with active and inactive sediment.

Correlations between UP^{PRM} and UP^{CBB}, and between UPC^{PRM} and UPC^{PRM} were highly significant ($P < 0.0001$) in all groups of samples. In the whole set of samples correlation coefficients were 0.82 and 0.91 for urinary proteins and for UPC, respectively; coefficients in the samples with active sediments were 0.96 for both proteinuria and UPC; in the samples with inactive sediments coefficients were 0.78 and 0.96 for protein and for UPC, respectively.

Statistical results of the method comparison study (including intercept and slope with 95% confidence intervals) obtained by Passing-Bablok regression analysis, and Bland-Altman biases with 95% limits of agreement obtained from UP and UPC ratio in the whole set of sample, in samples with active and with inactive sediments were shown in Table 8 and graphically displayed in Figures 8 and 9. Constant and proportional errors were found in all sets of samples, with the exception of UPC in inactive sediment set that yielded no constant bias.

The agreement in staging samples according to IRIS guidelines (Table 4) was defined as “good” in the whole set of samples (k coefficient =0.62), “moderate” for both active and inactive groups of samples (0.59 and 0.56 respectively).

Table 1 Median and range of UP, UC and UPC of the 120 samples included in the method comparison. Data of the whole caseload and of samples with inactive or active sediment are shown.

	All samples	Active sediment	Inactive sediment
UP^{PRM} (mg/dL)	28.9 (0.9-919.7)	40.3 (2.3-919.7)[a]	25.5 (0.9-345.3)
UP^{CBB} (mg/dL)	56.6 (2.8-614.8)	74.2 (8.9-595.0)[a]	48.2 (2.8-286.3)
UC (mg/dL)	162.0 (23.9-234.2)	152.9 (23.9-632.6)	158.2 (28.2-520.7)
UPC^{PRM}	0.17 (0.01-24.32)	0.28 (0.02-12.92)[b]	0.15 (0.01-6.97)
UPC^{CBB}	0.31 (0.03-17.41)	0.42 (0.09-14.95)[c]	0.22 (0.03-5.78)

UP^{PRM}, urinary protein measured with pyrogallol red-molybdate; UP^{CBB}, urinary protein measured with Coomassie brilliant blue; UC, urinary creatinine; UPC^{PRM} urinary protein-to-creatinine ratio measured with pyrogallol red-molybdate; UPC^{CBB} urinary protein-to-creatinine ratio measured with coomassie brilliant blue. Letters indicate which P value refer to comparison between samples with active sediment vs inactive sediment: a <0.05, b P <0.005, c P <0.01.

Table 1 Intercept and slope of Passing-Bablok tests and bias recorded in Bland–Altman tests (showed in Figure 8 and 9) of UP and UPC ratios measured with both methods for the whole set of sample and for active and inactive sets of samples. Cohen's k coefficients describing the concordance in classify samples according to International Renal Interest Society (IRIS) are also showed.

		Passing-Bablok		Bland-Altman	Cohen
		Intercept (95% CI)	**Slope (95% CI)**	**Bias (95% CI)**	**K coefficients**
All	UP	10.70 (6.67 to 14.91)	1.21 (1.10 to 1.33)	-17,82 (-7.50 to -28.14)	
	UPC	0.03 (0.02 to 0.05)	1.27 (1.18 to 1.43)	-0.11 (0.02 to -0.25)	0.62
Active	UP	13.01 (5.27 to 20.77)	1.14 (1.01 to 1.27)	-19.61 (4.72 to -43.95)	
	UPC	0.07 (0.03 to 0.10)	1.15 (1.04 to 1.30)	-0.2 (-0.06 to -0.34)	0.59
Inactive	UP	7.6 (0.39 to 15.44)	1.29 (1.05 to 1.61)	-17.68 (-12.6 to -22.77)	
	UPC	0.01 (-0.02 to 0.04)	1.49 (1.26 to 1.83)	-0.14 (-0.05 to -0.23)	0.56

UP, urinary protein; UPC, urinary protein-to-creatinine ratio

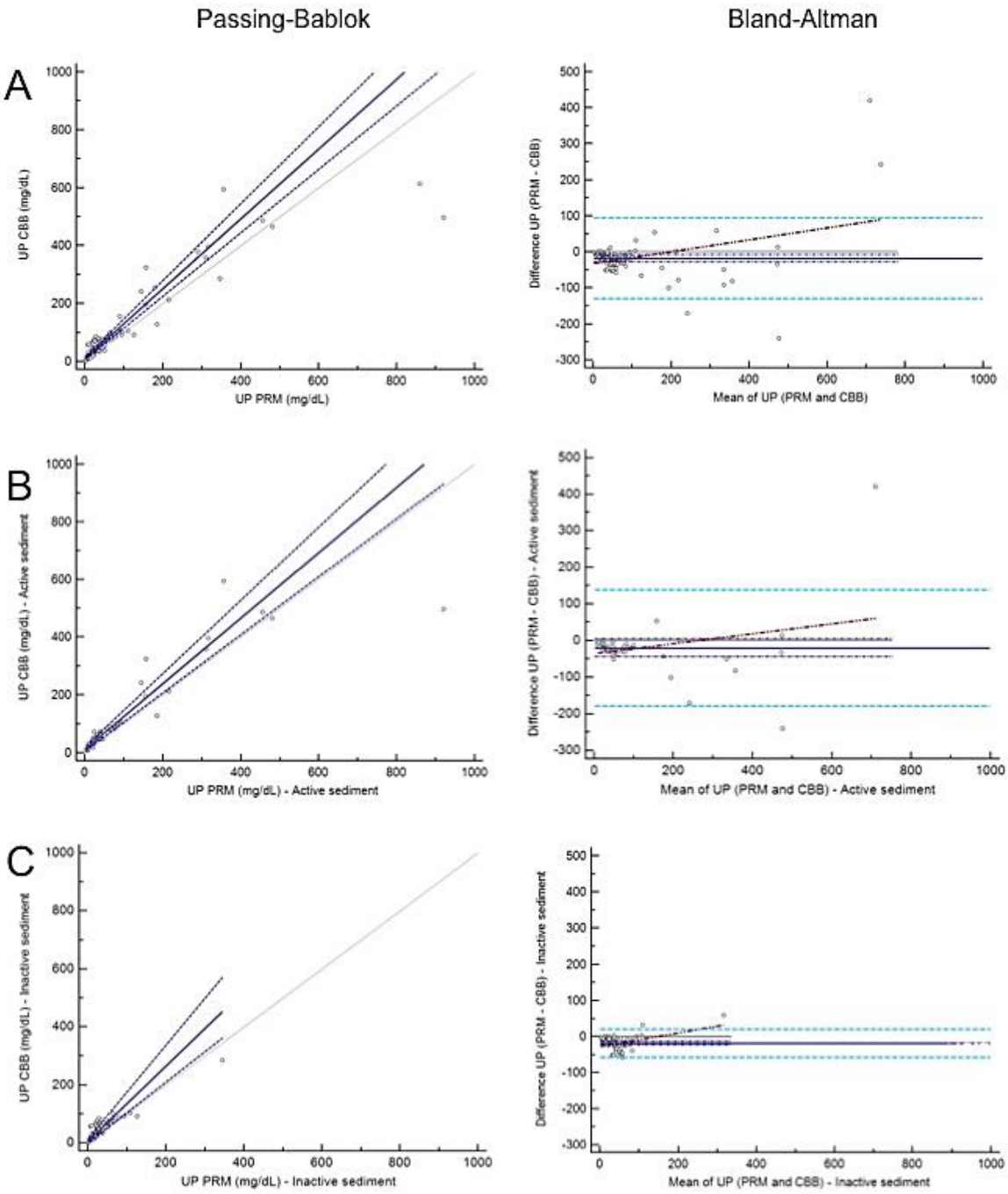


Figure 8 Passing-Bablok (left) and Bland-Altman plots (right) showing the comparison of urinary protein (UP) between Pyrogallol red molybdate (PRM) and Coomassie brilliant blue (CBB) obtained from 120 cats in whole set of sample (A) and for active (B) and inactive sets (C) of samples. In Passing-Bablok plots, the blue line is the correlation, the gray line shows best fit and the blue dotted lines represent 95% CI. In Bland–Altman plots, X axes represent the average between the two methods, and the Y axes the indicate the difference between PRM and CBB; the grey line shows the zero bias, the blue solid with the dashed blue lines represent the bias and 95% confidence interval (CI), respectively, the light blue dashed lines are the limits of agreement and the red dotted line is the regression line.

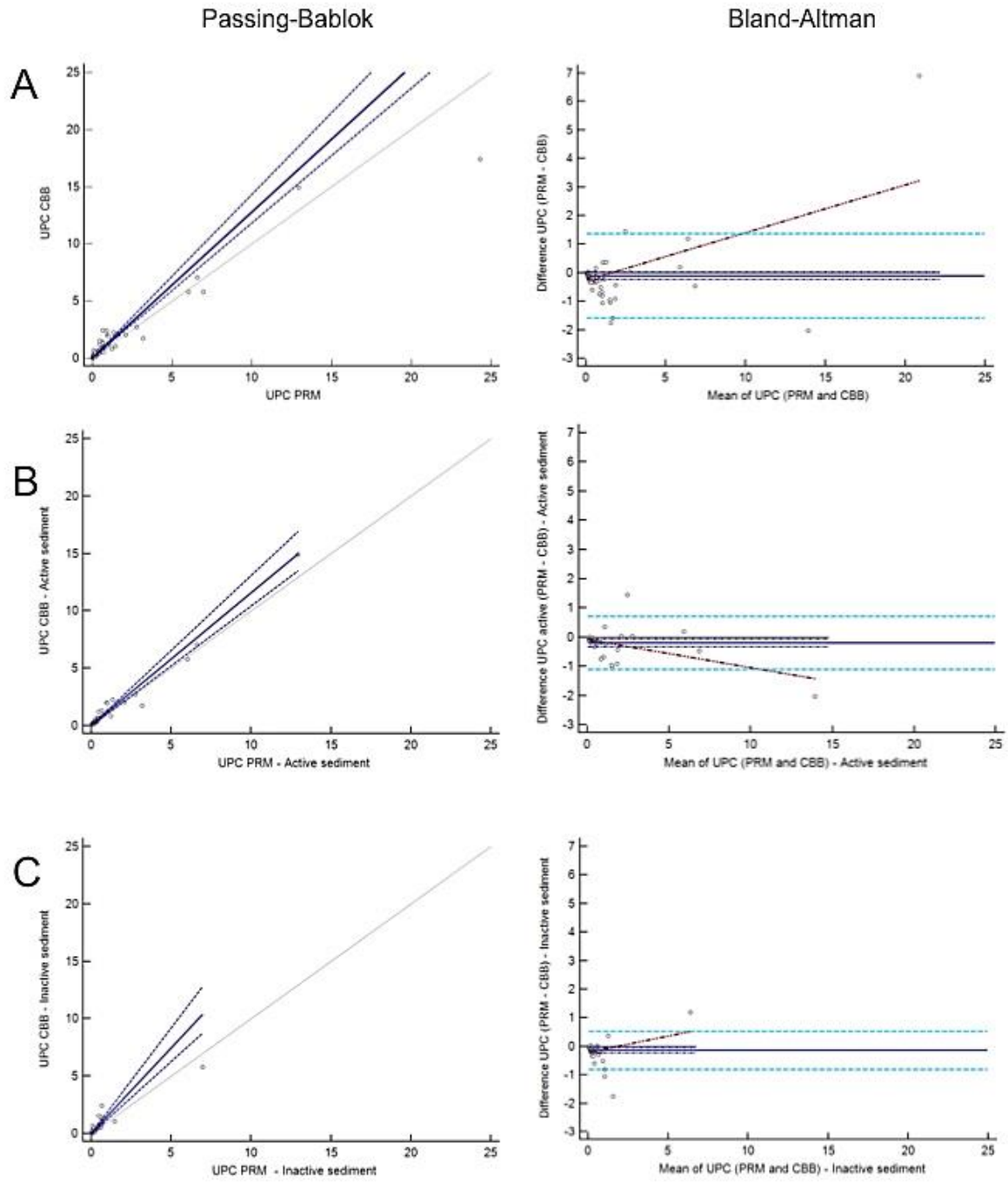


Figure 9 Passing-Bablok (left) and Bland-Altman plots (right) showing the comparison of urinary protein-to-creatinine (UPC) ratio between Pyrogallol red molybdate (PRM) and Coomassie brilliant blue (CBB) obtained from 120 cats in whole set of sample (A) and for active (B) and inactive (C) sets of samples. In Passing-Bablok plots, the blue line is the correlation, the gray line shows best fit and the blue dotted lines 95% CI. In Bland–Altman plots, X axes represent the average between the two methods, and the Y axes the indicate the difference between PRM and CBB; the grey line shows the zero bias, the blue solid with the dashed blue lines represent the bias and 95% confidence interval (CI), respectively, the light blue dashed lines are the limits of agreement and the red dotted line is the regression line.

Discussion

In this study, pre-analytical and analytical variables in quantification of feline urinary proteins and UPC ratio were evaluated in order to determine their potential effect on clinical decisions.

The dilution of urinary supernatants that is performed to measure urinary creatinine was shown to be a source of variability in dogs (Rossi et al., 2012).

In this study, although the range of linearity of the Jaffe method (0-20 mg/dL) was exceeded in many cases with 1:10 dilution, no difference was found between the three dilutions for urinary creatinine measurement or UPC ratios.

Feline urines generally have higher creatinine concentration (compared to dogs) due to the higher concentration capacity. Hence, the lower supernatants predilution (1:10) was expected to dilute urine not enough to lower creatinine concentration within the range of linearity of the methods. Interestingly, among the 25 samples, in two cases the 1:10 dilution yielded different IRIS stage when compared to the other two (1:20 and 1:100).

According to these results, all the 3 dilutions could be used for quantification of urinary creatinine in feline urine in order to obtain reliable UPC results. Therefore, in all the other test performed in this study, 1:20 dilution was chosen in order to adhere to the internal standard operative procedure.

The two methods for urinary protein quantification yielded CV values similarly to what already found in dogs (Rossi et al., 2012). A higher value was found with PRM for the sample with protein concentration close to the lower limit of the range of linearity (20 mg/dL) of the method. It's worth to note that the magnitude of CV of this sample could dramatically affect clinical decisions because it could potentially cause shift of the IRIS sub-stage for CKD. However, BP or P samples with low UP and UC are rare (3/120 cases in this study); therefore, the influence of high CVs at low protein concentration is negligible. The CBB method has the advantage to yield on average lower CV values compared to PRM but from a practical standpoint similar numbers of misclassifications were found in samples with UPC close to the two IRIS cut-off. Due to the magnitude of the intra-assay variability, in samples with UPC close to 0.2 and 0.4 it's advisable to interpret results with caution and to repeat measures of UPC over time in order to properly sub-stage feline patients affected by CKD. The inter-assay CVs found in this study were higher than the most common biochemical analytes (Harr et al., 2013) and could affect clinical decisions even more than intra-assay variability. However, because information about

biological variability of proteinuria in cats is not available, it's not known whether these inter-assay CV values could be considered acceptable.

The lack of statistical differences in storage at room and refrigerator temperatures and in repeated freezing-thawing cycles during the periods evaluated in this study suggested that measurement of the UP in cats may provide reliable results in these settings.

Some statistical differences were found storing samples at -20°C. UP^{PRM} tended to be higher at an intermediate checkpoint (T3w) in active sediment subset. UPC^{PRM} tended to be lower at late checkpoints in the whole and inactive sets of samples whereas tended to be higher at T3w in active sediment subset in a fashion similar to UP^{PRM}. These differences are difficult to explain from a biochemical point of view. A combination of sample dehydration (Rossi et al., 2012) and protein degradation (Kania et al., 2010) over time could theoretically have produced these peculiar patterns. Accordingly, urine supernatant evaporation was suspected to affect protein concentration in stored canine urines (Rossi et al., 2012) and proteins were shown to reduce in stored human urine samples (Osberg et al., 1990) (Schultz et al., 2000). Moreover, in a previous study in human urines it was shown that fragments of albumin were less able to react with wet biochemistry methods (Eppel et al., 2000). The different pattern in active and inactive sets could reflect the different content of proteases and protein substrates and fragments. Further studies using higher number of samples with active and inactive sediment and samples with known concentration of specific proteins are needed to confirm these hypothesis.

Differently to UP^{PRM} but similarly to UPC^{PRM}, UP^{CBB} yielded mild lower values at the late checkpoint (T12w) albeit the respective UPC values resulted stable. This different variation of UP at 12 weeks detected with CBB compared to PRM was not the result of different samples included in the two analytical evaluations. This could be stated because no significant difference was found between UP values at T0 of the two different analysis (both measured with CBB; data not shown). One possible explanation of the decrease of UP detected with only CBB could be that the possible denaturation or degradation of proteins during long period storage could produce less reactivity with CBB reagent (compared to PRM dye). This could be in accordance to previous results in human medicine (Eppel et al., 2000). Whether these processes are present and analytically significant in feline urine need further research. Another possible cause of the difference at 3 months between the two methods could be the presence in some samples of the freezing-induced sediments. This kind of sediment was demonstrated to generate in

urinary supernatants after freezing mainly in presence of higher urinary calcium concentration and acid urine, and it was shown to lower the protein concentration (Saetun et al., 2009). However, in this study samples were vortexed after thawing and therefore the effect of such sediment on these results was probably unlikely.

Taken together, differences at -20°C found in this study were more important from a statistical standpoint than from an practical point of view because at all checkpoints, sets of samples and method used UPC values were within the inter-assay CV. Therefore, results could be considered stable in all the cases from a clinical point of view.

Among the several commercially available automated methods for measurement of urinary proteins, the two most used dye-binding methods were evaluated in this study. Constant and proportional errors were demonstrated in the whole set of samples and agreement did not improve neither in samples with inactive sediment, where UPC values gain clinical significance. Similar results have been previously reported in a smaller group of feline samples, comparing different analytical assays (specifically, colorimetric pyrocatechol violet dye-binding says and turbidimetric benzethonium chloride assay) (Fernandes et al., 2005). In this study, CBB yielded higher protein concentration and in turn UPC ratios when compared to PRM. Similar positive bias of CBB was demonstrated in dogs for quantification of urinary proteins (Rossi et al., 2016) and total protein in cerebrospinal fluid (Riond et al., 2014). Conversely, in human urine CBB tended to yield lower protein concentration when compared to PRM (Marshall and Williams, 2000). One important cause of discrepancy between these two methods was shown to be the different responses of dyes to different types of proteins. For example, both methods were shown to constantly underestimate globulin when compared to albumin (Marshall and Williams, 2000; Riond et al., 2014; Nishi et al., 1985; Watanabe et al., 1986). Samples included in this study probably presented a large variability of protein types due to the different underlying diseases and this variability could persist also within the inactive and active sets of samples. This heterogeneity reflected the actual variability of protein patterns in samples commonly assayed in diagnostic laboratories and allowed to quantify analytical variability from a practical point of view. Analysis of the protein content of urine samples was beyond the aim of this study and whether the agreement between methods is different in specific diseases or protein patterns need further research. Because of the different response to different proteins, the use of the same standard for calibration of different methods and the use of mixed proteins instead of a single protein (such as albumin) as standard solution were proven to improve the agreement between methods (Marshall and

Williams, 2000). The two methods evaluated in this study were calibrated with the standards provided by the manufactures. The use of the original standards had the aim to evaluate the actual variability that could be found between laboratories. Further studies are needed in order to evaluate whether the agreement improves using the same standard, possibly composed by mixed proteins or feline urinary proteins.

The concordance in classifying samples according to the IRIS staging was never in the higher category of classification according to the Cohen's k coefficients (i.e. "very good"). Although concordance in active and inactive subsets of samples was defined moderate and lower than that found in whole set of samples, k coefficients were very close in magnitude and concordance in the three sets of samples could be considered similar. It can be stated that these low concordances were the results of the tendency of CBB to misclassify samples in higher stages, as discussed above. On this regard, it's worth to note that in some cases the magnitude of the bias was so high that samples were graded as non proteinuric with PRM and proteinuric with CBB. These patients would experience different diagnostic approaches and possibly different therapies. Taken together, the results of the method comparison study pointed out that the use of the same laboratory and the same method should be recommended in monitoring patients over time and the comparison of results between different laboratories should be avoided. Moreover, the use of external reference intervals (as determined by IRIS) could enhance the clinical effect of analytical variability. Therefore, according to these results, the use of laboratory specific reference interval, as suggested in human medicine (Dube et al., 2005), or the improvement of the IRIS cut-off in relation to the different methods (Jefferey, 2017) should be advocated.

Conclusion

In conclusion, UPC can be considered relievable when stored up to 6 hours at room temperature or 1 week at refrigeration temperature; moreover, no effect of 4 freeze-thaw cycles was found but, when frozen samples are used, UPC should be interpret with caution in case of storage longer than 4 weeks, given the tendency to reduce in magnitude. Dilution of urine to quantify creatininuria did not appear to affect UPC and a dilution between 1:10 and 1:100 could be used.

Both PRM and CBB methods were precise but samples with UPC close to the cut-off of IRIS substaging should be carefully interpreted to avoid misclassification. Intrinsic difference between analytical methods resulted in inaccuracy and suboptimal concordance in classifying samples according to IRIS substaging. This disagreement could affect clinical decisions, make questionable the comparison of UPC results between different laboratories, and have significant impact in substaging cats affected by CKD, given the strict cut-off recommended in published guidelines in which the method of choice is not indicated.

2. Effects of hematuria and pyuria on feline proteinuria

Introduction and aims

The interpretation of proteinuria in cats with CKD requires the exclusion of extra-renal source of protein (Lee et al., 2005).

Urinary tract inflammation and hemorrhage are the main causes of post-renal proteinuria (Grauer, 2011) and are possible concurrent finding in CKD (Martinez-Ruzafa et al., 2012).

In dogs, proteinuria significantly increased only in case of macroscopic hematuria and was more likely to be high with concurrent hematuria and pyuria. (Vaden et al., 2004)

No information is available about the effect of such conditions on UPC in feline urinary samples. Therefore, the aim of this study was to determine whether the presence of hematuria or pyuria affects the urinary protein concentration and UPC ratio in feline urine

The aim of this study was to determine whether the presence of hematuria or pyuria affects the urinary protein concentration and UPC ratio in feline urine.

Materials and Methods

Data collection

The electronic database of the internal medicine unit of Veterinary Teaching Hospital was retrospectively searched for urinalysis of feline patients collected between January 2013 until May 2017.

Each record of the database corresponds to a sample obtained from client owned cats presented at our institution for routine health screening.

Records of patients without measurement of serum creatinine the same day of urinalysis or the day after were excluded.

Records with incomplete urinalysis data, samples lacking UPC ratio measurement and samples with unspecified method of collection or whose method of collection was different from cystocentesis and spontaneous micturition after manual collection (Vihena et al. 2015) were excluded. In the event that a patient had one or more urine samples collected within 1 month, only the first record was included and the other record (or records) was excluded.

For each sample, serum creatinine, UP, UPC ratio, USG, and semiquantitative evaluation of urinary red blood cells (RBC) and white blood cells (WBC) were recorded and used for analysis.

Analytical procedures

During the study period, the same standard operative procedures (SOPs) for urinalysis were performed in our lab, between 3 and 5 hours after collection. Specifically, urine specific gravity (USG) was determined by a handheld refractometer (Clinical Refractometer, model 105, Sper Scientific, Scottsdale, AZ, USA). In order to perform sediment examination, five mL of urine were transferred from the syringe or the urine container to a sterile conical tube). Tubes were than centrifuged at 1250 rpm for 5 minutes (450G) (Hermle Z300, Labnet international, Edison, NJ, USA) and 4.75 mL of supernatant was removed by suction using a dispensable pipette according to current guidelines (ECLM, 2000) in order to avoid loss of sediment and supernatant contamination by elements of the sediment and it was transferred in another tube.

Sediments were resuspended in the remaining supernatant and 50 µL were placed on a slide, covered with a microscope coverslip (24x32 mm) and evaluated microscopically al 100x (low power field, LPF) and 400x (high power field, HPF) magnifications. Urinary RBC and WBC were countered and reported as estimation of the mean number per high power field (HPF, 400x).

Hematuria and pyuria were defined as >5 RBC/HPF and >5 WBC/HPF, respectively (Callen et al., 2016). Samples with both <5 urinary RBC and WBC/HPF were classified as inactive.

On the suctioned supernatant, UP was measured using Pyrogallol red molybdate (PRM) method and UC were measured with modified Jaffe method using an automated biochemical analyzer (Cobas Mira, Roche Diagnostics, Basel, Switzerland). Serum creatinine was measured with the same biochemical method.

Study design

To the study purposes, a case-control study was performed separately for both hematuria and pyuria. Case-control comparison was applied in order to normalize the effect of other factors affecting proteinuria such as renal diseases, or pyuria on samples with hematuria and hematuria on samples with pyuria,

Concerning hematuria, all samples with >5 RBC/HPF were grouped together (group "RBC") and each sample was matched with an other sample having <5 RBC/HPF, to form the control group labeled "control-RBC". The match were based on serum creatinine (both samples with lower or higher 1.6 mg/dL, corresponding to the upper limit of reference interval of our laboratory), USG (both samples with lower or higher 1035, considered the limit of the concentration ability in cats) (Reppas and Foster, 2016) and pyuria (both samples with lower or higher 5 WBC/HPF)
Concerning pyuria, all samples with >5 WBC/ HPF were grouped together (group "WBC") and the control group (group "control-WBC") was similarly created matching each sample of the former group to an other sample having <5 WBC/HPF, on the basis of serum creatinine, USG and hematuria (categorized as higher or lower 5 RBC/HPF).

A second evaluation was performed only on the subset of samples with USG >1035 and serum creatinine <1.6 mg/dL. These patients were considered to present adequate renal function and therefore kidney disease and renal proteinuria were deemed less likely. Samples without pyuria (<5 WBC/HPF) were grouped according to the level of RBC/HPF as follow: <5 RBC/ HPF, 5-20 RBC/HPF and >20 RBC/HPF. A similar analysis on samples without hematuria (<5 RBC/HPF) was not possible since in our database only five samples had concurrently >5 WBC/HPF, <5 RBC/HPF, USG >1035 and serum creatinine <1.6 mg/dL.

Statistical analysis

Statistical analysis was performed with commercially available software (GraphPad Prism 5.0; GraphPad Software, San Diego, CA, USA) and a P value <0.05 was considered statistically significant. Distribution of variables was assessed by Kolmogorov-Smirnov test. Mann-Whitney U test was used to compare UP, UPC ratio, between hematuria and pyuria groups with the respective control groups. Kruskal-Wallis test and Dunn post test were used to compare UP and UPC between groups divided according to the level of RBC/HPF.

Results

Two hundred and six complete urinalysis, associated with serum creatinine measurement, were retrieved. RBC were <5/HPF in 125 samples, between 5 and 20/HPF in 22 samples

and >20/HPF in 59 samples. WBC were <5/HPF in 180 samples, between 5 and 20/HPF in 16 samples and >20/HPF in 10 samples.
Serum creatinine ranged from 0.51 to 10.93 mg/dL (median =1.63 mg/dL), UP ranged from 1.1 to 480.0 mg/dL (median =23.65 mg/dL), UPC ratio from 0.01 to 10.20 (median =0.15), USG from 1007 to 1096 (median=1033).

The case-control analysis revealed significantly higher UP (P=0.0048) and UPC (P=0.0433) in “RBC” group compared to “RBC-control” group whereas no significant difference were found between “WBC” and “WBC-control” groups (Figure 10).
In samples without pyuria, USG >1035 and serum creatinine <1.6 mg/dL grouped according to the levels of RBC/HPF, UP (median: 45.5 mg/dL; min-max: 12.6-367.8 mg/dL) and UPC (median: 0.19; min-max: 0.04-1.78) were significant higher (P=0.0035 and 0.0339, respectively) in “>20 RBC/HPF” group than in “<5 RBC/HPF” group (UP: median: 28.2; min-max: 1.0-352.0; UPC: median: 0.10; min-max: 0.01-1.42), whereas in “5-20 RBC/HPF” group neither UP (median: 30.3; min-max:13.4-93.0) nor UPC (median: 0.06; min-max: 0.05-0.95) were different (Figure 11). According to IRIS staging for proteinuria (Elliott and Watson, 2016), in “>20 RBC/HPF group” 4 samples (20%) were defined proteinuric (UPC ratio > 0.4) and 9 samples (45%) borderline proteinuric (UPC ratio between 0.2 and 0.4).

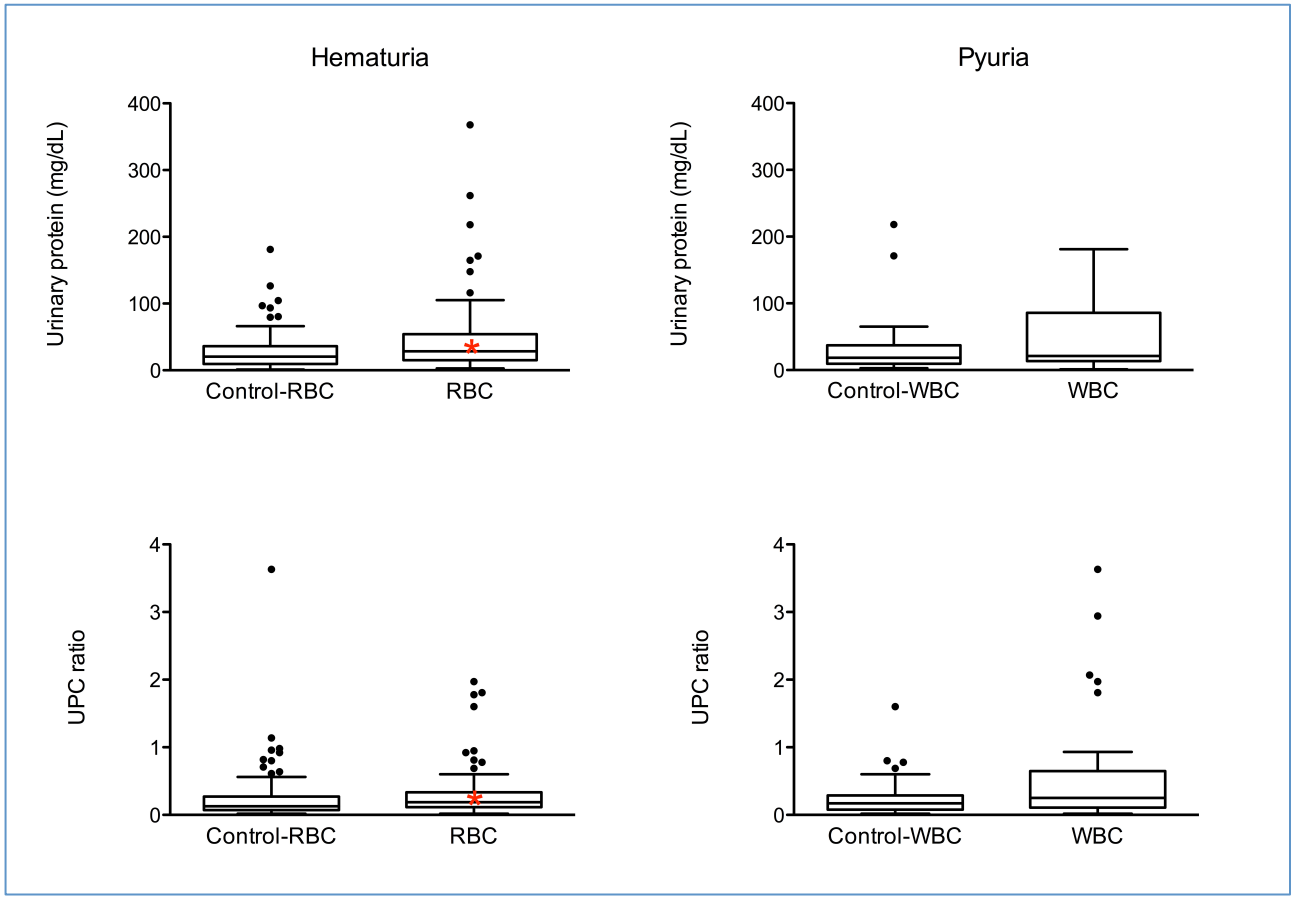


Figure 10 Distribution of urine protein (UP) concentration and urinary protein-to-creatinine (UPC) ratio measured in hematuria (83 samples) and pyuria (31 samples) groups and in the respective control groups (83 and 31 samples, respectively). Box plots represent the interquartile range (IQR), and the horizontal line represents the median value. Whiskers extend to the furthest observations within ± 1.5 IQRs of the first or third quartile. Dots indicate values > 1.5 IQR. Red asterisks within boxes indicate a significant (P < 0.05) difference compared with control group

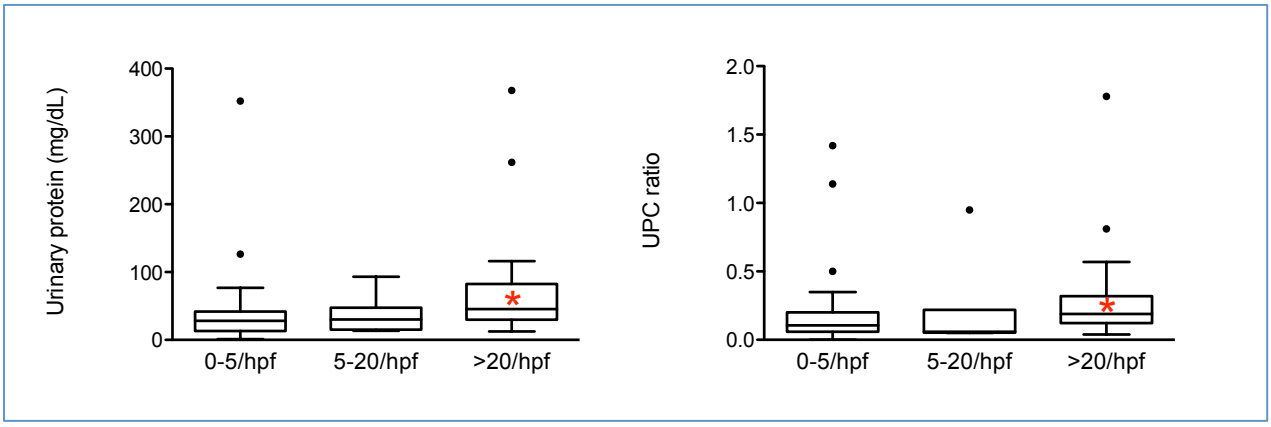


Figure 11 Distribution of urine protein (UP) concentration and urinary protein-to-creatinine (UPC) ratio measured in samples grouped according to RBC<5 RBC/hpf (56 samples), RBC between 5 and 20 RBC/hpf (8 samples) and >20 RBC/hpf (20 samples) without pyuria, with serum creatinine < 1.6 mg/dL and USG >1035.Box plots represent the interquartile range (IQR), and the horizontal line represents the median value. Whiskers extend to the furthest observations within ± 1.5 IQRs of the first or third quartile. Dots indicate values > 1.5 IQR. Red asterisks within boxes indicate a significant (P < 0.05) difference compared with RBC<5 RBC/hpf group.

Discussion

According to the results of this study, the presence of RBC in urine affects UP and in turn UPC ratio.

Higher UPC values were recorded with higher amount of RBC (>20 RBC/HPF). In dogs, significant increase of UPC was demonstrated only with gross hematuria (>250 RBC/HPF). In that study RBC from peripheral blood were added to urine samples and were serially diluted whereas in our study field samples were included (Vaden et al., 2004).

Although the most probable source of hematuria in our samples were hyatrogenic hematuria during cystocentesis, other causes of hematuria such as mucosal bleeding due to coagulation disorder or lesions such as neoplasia could not be excluded, albeit unlikely.

Therefore, the different study design and the different protein patterns present in urine could explain the difference between these results and those found in dogs. Further studies are necessary in order to determine whether different source of blood change differently UPC. Interestingly, in the set of samples without pyuria (and serum creatinine <1.6 and USG>1035) grouped according to the level of RBC/HPF, the sample with the highest UPC ratio (UPC=1.78) had macroscopic hematuria. Hence, it could be speculated that UPC could proportionally increase as RBC increase.

Conversely to hematuria, pyuria did not increase the UP and UPC ratio. This is in accordance to what found in dogs, where UPC was higher in pyuric samples only when associated with hematuria (Vaden et al., 2004). Few samples had concurrent pyuria and laboratory parameters compatible with adequate renal function (i.e. creatinine within RI and USG > 1035). This lack could be due the concurrent presence of CKD and UTI in our population, as already reported, (Mayer-Roenne 2007). Moreover, it could be due to the tendency to not measure UPC in samples with pyuria and UTI and that bias could be considered a limitation of this retrospective study. Therefore, further prospective studies are warranted to better evaluate the effect of pyuria on UP and UPC.

Although the study was designed to attempt to exclude the main causes of pathologic proteinuria except hematuria and pyuria, the presence of renal or prerenal diseases in some samples could not completely excluded but was deemed unlikely.

Conclusion

In conclusion, hematuria could affect UP and UPC in cats and the effect appeared to be proportional to RBC concentration. From a practical point of view, diagnosis of renal proteinuria and substaging of proteinuria in patients affected by CKD according to IRIS should be avoided in samples with >20 RBC/HPF or interpreted with caution. Further prospective studies are necessary to confirm the minor effect of pyuria on proteinuria reported in this study.

3. Comparison between the concentration of creatinine and symmetric dimethylarginine (SDMA) in blood of Holy Birman cats

Introduction and aims

Holy Birman cats have a physiologically high concentration of serum creatinine and urea compared with the feline general population (Reynolds et al., 2010; Paltrinieri et al., 2014). Moreover, Holy Birman cats have been reported as prone to develop FIP (Pesteanu-Somogyi et al., 2006), a disease on which renal functions are frequently altered (Pedersen, 2014), and may be affected by hereditary polycystic kidney disease (PKD), and therefore in this breed clinical and laboratory investigation may be frequently performed also in younger cats. In all these clinical scenarios, the presence of renal disease risks to be overestimated in Holy Birman cats, due to their physiologically high creatinine concentration: the detection of high serum creatinine levels often induces owners and vets to perform unnecessary and stressful clinical and laboratory workup to exclude the presence of CKD.

Recently, symmetric dimethylarginine (SDMA) has been proposed as a novel marker for an early diagnosis of CKD in cats (Jepson et al., 2008; Braff et al., 2014; Hall et al., 2014a). As for creatinine, SDMA is excreted by the kidneys and therefore its serum concentration increases during CKD and this increase seems to occur earlier than the increase of creatinine in cats (Hall et al., 2014a).

Based on our knowledge on the metabolism of SDMA (Fleck et al., 2003), which is different from that of creatinine (Braun et al., 2003), it is very likely that despite the high creatinine concentration, in clinically healthy Holy Birman cats SDMA have the same concentration than in the general feline population.

Therefore, SDMA may be proposed as a marker to monitor the health status in this feline breed, thus reducing the number of unnecessary samplings and testing to exclude CKD.

The aim of this study was to assess whether clinically healthy Holy Birman cats (as a representative breed with high creatinine concentration) have normal SDMA concentrations, thus allowing, in this breed, a better evaluation of renal function compared with creatinine, and to define the breed-specific reference interval for SDMA, if needed.

Materials and Methods

Caseload

This study was done on blood samples collected from clinically healthy Holy Birman cats, randomly collected through collaboration with the Italian association of Birman cats breeders, that were subjected to regular veterinary visits, that included routine bloodwork, urinalysis, abdominal ultrasound and genetic screening for hereditary disorders. The inclusion criteria for Birman cats were the absence of any abnormality detected by physical examination and diagnostic imaging (see below), and the absence of laboratory changes compared with breed specific reference intervals.

As a control group, blood from cats belonging to breed other than Holy Birman, and collected at our Institution during annual wellness visits, before elective surgery (e.g. castration, dental scaling) or within clinical examination for diseases not associated with decreased glomerular filtration rate (mostly non-metastatic tumors). The inclusion criterion for these control cats was a serum concentration of creatinine lower than 1.6 (i.e. the threshold value considered as normal by the International Renal Interest Society) (IRIS, 2015x), the absence of other abnormalities in routine blood and urine analysis.

The study was approved by the Institutional Animal Care and Use Committee (approval number: 2/2016).

In both groups, samples from cats younger than 6 months or affected by diseases potentially influencing the glomerular filtration rate, and samples presenting gross abnormalities consistent with hemolysis or lipemia were excluded from the study.

Samples were collected from the cephalic vein by the referring veterinarians upon informed consent from the owners, placed in tubes without anticoagulant, centrifuged and frozen at -20°C to be periodically sent to our Department. At our Department, samples were thawed, and used to perform a basic panel of biochemical tests to exclude the presence of laboratory abnormalities. This panel included the measurement of creatinine, urea, total protein, albumin, ALT, GGT, cholesterol, and was performed using an automated spectrophotometer (Cobas Mira, Roche, Basel, Switzerland) using reagents provided by Hagen Diagnostica (Arezzo, Italy) The remaining serum samples sent to IDEXX laboratories to perform SDMA analysis. SDMA was measured in duplicate or, when the difference between the two measurements exceeded 2.0 µg/dL, in triplicate. The mean values of the replicas recorded in each cat were included in this study.

Statistical analysis

Results regarding age, and creatinine and SDMA recorded in Holy Birman and in from cats of other breeds were compared using a non parametric t-test for unpaired samples (Mann U Withney) using statistical software (Analyse-it version 2.21, Analyse-it Software Ltd, Leeds, UK). The same software was used to assess, using the Pearson's chi square test, the possible differences in the proportion of cats with increased SDMA compared with the reference intervals among Holy Birmans and among cats of other breeds.

In order to assess whether the concentration of SDMA in Holy Birman cats falls within the reference intervals for the general population of clinically healthy cats determined by the producer of the kit, we applied the method of transference of reference intervals (NCCLS, 2010), that validates pre-existing reference intervals if less than 10% of 20 randomly selected results. A breed-specific reference interval was then established for Birman cats using an Excel spreadsheet with the Reference Value Advisor (v. 2.0) set of macroinstructions (Geffré et al., 2011), that, according the recommendations of the IFCC-Clinical and Laboratory Standards Institute (NCCLS, 2010), performs descriptive statistics, tests of normality (Anderson–Darling with histograms and Q–Q plots and Box–Cox transformation) and outlier analysis. Both Dixon–Reed and Tukey tests were used to detect outliers, that were retained unless clear reasons to classify them as aberrant observation were found, as recommended by the ASVCP guidelines (Friedrichs et l., 2012). Reference intervals were calculated using a non-parametric bootstrap method, that calculated also the 90% confidence interval of reference limits. Linear regression was used to assess the possible influence of age on reference intervals of SDMA in Holy Birman cats.

Results

Group composition

The study population was composed by 101 cats, which fulfilled the inclusion criteria.

Of these, 50 were Holy Birman cats (21 female, 6 spayed female, 17 male, 6 neutered male; age range: 6 months to 13 years, median age: 4 years).

The remaining 51 cats belong to 8 different breeds (39 domestic shorthair, 3 Persian, 2 Exotic shorthair, 2 Maine Coon, 2 British shorthair, 1 Devon Rex, 1 Scottish fold, 1

Siberian cat). This group was composed by 10 female, 21 spayed female, 4 male, 16 neutered male (age range: 6 months-17 years, median age: 9 years).
The age of Holy Birman cats was significantly lower than the age of cats of other breeds (P<0.001).

Creatinine and SDMA concentration

Both creatinine (mean ± SD: 1.58 ± 0.36 mg/dL; median: 1.47 mg/dL) and SDMA (12.2 ± 2.8 µg/dL; 12.0 µg/dL) were significantly higher (P<0.001 for both) in Holy Birman cats than in cats of other breeds (1.17 ± 1.16 mg/dL; 1.16 mg/dL for creatinine, and 10.1 ± 2.5 µg/dL; 9.5 µg/dL for SDMA). However, the distribution of data (figure 12) revealed a higher degree of overlapping between groups for SDMA than for creatinine.

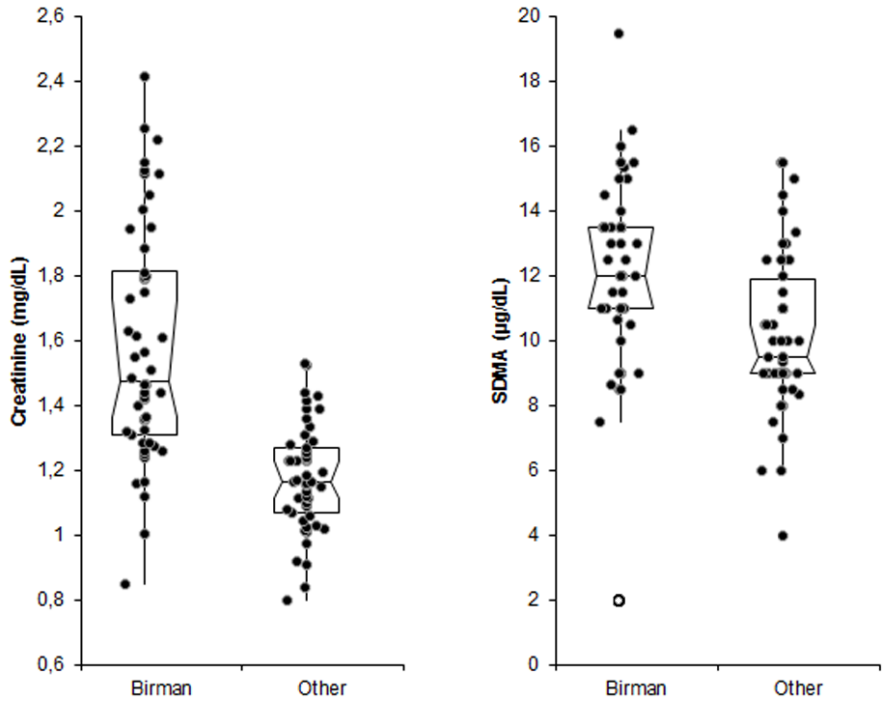


Figure 12 Distribution of results of creatinine and SDMA recorded in Holy Birman cats and in cats of other breeds. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule. Each black dot corresponds to an individual value. Far outliers are indicated with the open circles.

No significant gender-related differences were found for SDMA for Holy Birman cats (P=0.963) or for cats of other breeds (P=0.602). Similarly, no significant differences by age were recorded in the two groups (P=0.684 and P=0.560, respectively).
The analysis of individual data (table 11) revealed that in 20/50 Holy Birman cats (40.0%) the concentration of serum creatinine was higher than 1.60 mg/dL, i.e. the threshold

recommended to classify cats as affected by CKD stage II (Elliot and Watson, 2016). In 5 out these 20 cats the deviation from the IRIS threshold was lower than 10%, which may be consistent as an acceptable analytical error for creatinine (Ricós et al., 1999) while in the remaining 15 cases the deviation from the threshold largely exceeded 10%. However, all the values were below the upper limit of the breed-specific reference interval established for Holy Birman cats in a previous study (2.5 mg/dL) (Paltrinieri et al., 2014).

Table 9 Individual data recorded in Holy Birman cats and in cats of other breeds. In bold, creatinine values higher than the threshold recommended by the international renal interest society (IRIS)[4] to classify cats in stage II of renal disease, and SDMA values higher than the upper reference limit recommended by the producer of the test and included in the new IRIS stagin system.[4]

Holy Birman Cats				Cats of other breeds			
Cat	Age	Creatinine (mg/dL)	SDMA (µg/dL)	Cat	Age	Creatinine (mg/dL)	SDMA (µg/dL)
1	12	**1.89**	13.5	1	1	1.05	11.0
2	4	1.32	10.0	2	0.5	1.26	14.0
3	8	**1.62**	13.5	3	0.5	0.92	12.5
4	1	1.29	14.0	4	12	0.84	6.0
5	2	1.28	11.0	5	2	0.98	9.0
6	1	1.12	2.0	6	1	1.01	**15.5**
7	5	**1.95**	**15.5**	7	1	1.02	9.5
8	1	1.24	12.5	8	8	1.02	12.5
9	4	1.37	10.7	9	10	1.03	7.5
10	2	1.49	13.5	10	12	1.06	8.0
11	4	1.40	11.0	11	12	1.07	9.3
12	1	1.01	7.5	12	12	1.09	7.0
13	2	**1.80**	**15.3**	13	9	1.10	**15.5**
14	2	1.26	11.0	14	16	1.10	9.5
15	3	1.42	13.5	15	1.5	1.12	10.0
16	2	1.47	**14.5**	16	5	1.12	8.5
17	8	1.36	9.0	17	2	1.12	4.0
18	9	**2.11**	11.0	18	2	1.14	9.0
19	4	1.57	8.7	19	3	1.15	10.0
20	6	1.55	9.0	20	7	1.16	8.0

21	7	1.26	10.5	21	4	1.16	10.0
22	11	0.85	8.5	22	9	1.16	10.5
23	11	1.29	8.5	23	5	1.17	9.0
24	11	1.43	9.0	24	2	1.17	9.0
25	10	1.17	**16.5**	25	14	1.17	9.0
26	3	**2.26**	**15.0**	26	10	1.19	9.5
27	1	**2.12**	13.0	27	9	1.20	8.5
28	5	1.51	13.5	28	10	1.23	13.3
29	2	1.47	12.0	29	8	1.23	11.0
30	2	**1.73**	13.0	30	12	1.23	8.3
31	1	1.44	11.5	31	6	1.23	10.5
32	13	1.44	13.0	32	12	1.24	11.5
33	5	**2.22**	**15.5**	33	11	1.24	9.0
34	1	1.25	11.0	34	2	1.26	**14.5**
35	2	**2.13**	**19.5**	35	10	1.27	9.0
36	1	**1.75**	12.0	36	17	1.31	12.5
37	1	1.33	**15.5**	37	13	1.34	10.5
38	1.5	1.16	12.5	38	10	1.42	12.5
39	1.5	**1.95**	12.0	39	4	1.43	9.0
40	4	**2.01**	13.5	40	15	1.53	11.0
41	8	**1.81**	**15.0**	41	11	1.29	13.0
42	6	**2.05**	13.5	42	11	1.36	9.0
43	1.5	1.36	12.0	43	14	1.03	9.0
44	6	**2.42**	**16.0**	44	9	1.28	6.0
45	6	**1.61**	11.0	45	11	1.39	**15.0**
46	7	**1.80**	11.5	46	7	0.8	12.0
47	4	**2.15**	13.5	47	6	1.44	10.0
48	5	1.31	12.0	48	14	1.08	9.0
49	5	**1.63**	12.0	49	10	1.39	8.0
50	5	**1.79**	9.0	50	8	0.91	9.0
				51	14	1.53	13.0

The concentration of SDMA was higher than the upper limit of the pre-existing reference interval (14 µg/dL) in 10/50 Holy Birman cats (20.0%), and in 4 cats belonging to other breeds (7.8%). The proportion of individuals with increased SDMA compared with the reference intervals was not significantly different (P=0.100). In 7 of the 10 Holy Birman cats with increased SDMA (14.0% of the total number of Birman cats included in this study), and in all the 4 cats of other breeds with increased SDMA, the deviation from the reference intervals did not exceeded 1.5 µg/dL (approximately the 10% deviation that is consistent with the intrinsic variability of the method determined by the CVs recorded in duplicate or triplicate measurement). Also in this case the proportion of cats with a deviation lower than 10% compared with the reference intervals was not significantly different in Holy Birman cats compared with cats of other breeds (P=0.374). A deviation higher than 1.5 µg/dL from the upper reference limit was found only in 3 Holy Birman cats (6.0% of the total number of Holy Birman cats included in the study), whose SDMA concentration was 16.0, 16.5, 19.5 µg/dL, respectively. Two of these 3 cats had also creatinine values higher than 1.6 mg/dL. Despite in all the cats of other breeds the deviation from the upper reference limit was lower than 1.5 µg/dL, no significant differences (P=0.076) were found between Holy Birman cats and cats of other breeds with a deviation higher than 1.5 µg/dL from the upper reference limit.

However, based on these results, the transference method for reference interval did not validate, in Holy Birman cats, the reference interval generated in the general feline population. Therefore, a breed specific reference interval was generated for SDMA using the statistical approach recommended by the ASVCP guidelines. This approach identified a single outlier that, however, was in the lower and not clinically significant part of the dataset (Figure 12), and defined a reference interval ranging from 3.5 µg/dL (90% confidence interval: 2.0-8.5 µg/dL) to 18.7 mg/dL (90% confidence interval: 15.5-19.5 µg/dL).

Discussion

The results of this study confirmed the physiologically high serum concentration of creatinine in Holy Birman cats, already reported in previous studies (Reynolds et al., 2010) (Paltrinieri et al., 2014), either in terms of cats with creatinine values higher than the IRIS threshold for stage II[4] or in terms of magnitude of the increase compared with this threshold.

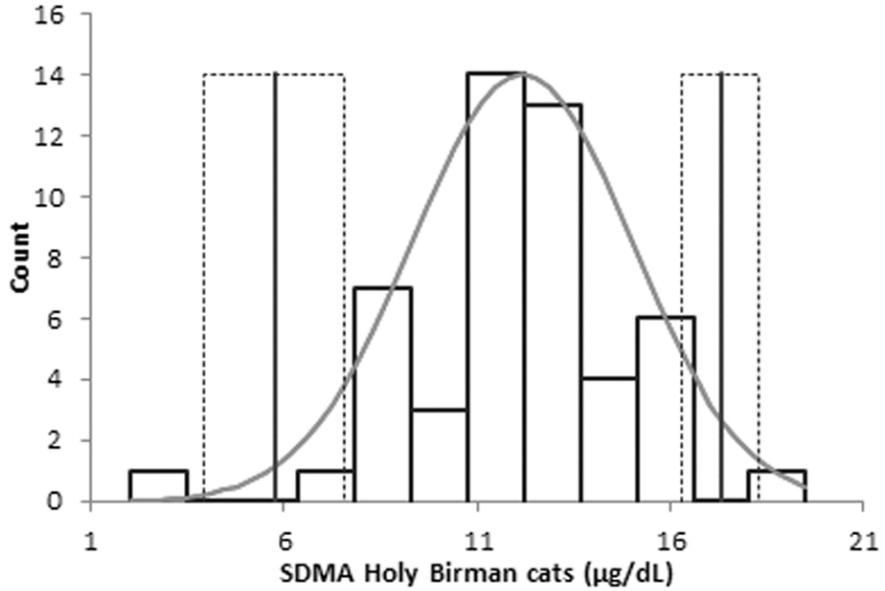


Figure 13 Distribution of data regarding SDMA concentration in Holy Birman cats. The bars indicate the frequency of each point value. Vertical solid lines indicate the limits of the reference intervals (dotted lines represent the 90% CI of each limit). The grey line indicates the fitted distribution, which depicts a Gaussian curve.

Also the concentration of SDMA was also increased in some Birman cats. Theoretically, cats with increased SDMA should be classified, according to the most recent version of IRIS guidelines (Elliot and Watson, 2016) in stage I when creatinine is <1.6 mg/dL or in higher stages when creatinine is >1.6. However, in most cases, the increase recorded in Holy Birman cats was quantitatively minimal and therefore, this increase may be imputable to the analytical variability of the method, determined by the analysis of duplicate analysis of SDMA on the samples included in this study (IRIS, 2015x), coupled with the normal variability compared with reference intervals, that actually include only 95% of the general population (Geffré et al., 2009). An increase of similar magnitude was found also in a similar proportion of cats of other breeds, as well as in a previous study in dogs (Nabity et al., 2015), on which the maximum values of 16 µg/dL, despite of an upper reference limit of 14 µg/dL, was wound in healthy subjects. This confirms that such an increase in magnitude is likely non relevant on a biological/diagnostic point of view. In order to exclude that these cats have an ongoing CKD, not diagnosed by serum creatinine due to its lower sensitivity compared with SDMA (Hall et al., 2014a), repeated measurements over time should performed since only persistent increases of SDMA should be considered consistent with an ongoing CKD (Elliot and Watson, 2016). However, as a further support to the hypothesis that the slight increases recorded in some cats were likely dependent on

the intrinsic variability of the method, in both breed groups these changes were found either in cats older than 8 years, that are potentially at risk of CKD (Elliot and Watson, 2016), and on which SDMA may be an earlier marker of CKD than creatinine (Hall et al., 2014a) (Hall et al., 2014b), or in younger cats.

Despite the absence of statistical differences in the proportion of cats with increased SDMA, it is worth to note that in three cats (6% of Holy Birman cats) increases of SDMA concentration not attributable to the analytical variability were found, with an SDMA value particularly high in one of these. All these three cats were clinically healthy both at the time or sampling and during a follow up of 9 months, and did not have other laboratory abnormalities except for a concentration of serum creatinine higher than the IRIS threshold in 2 cases. This confirms the risk of overestimation of CKD in Holy Birman cats: although the lack of clinical signs over time demonstrate that these cats are likely healthy, based on laboratory results alone it is impossible to determine whether these two cats are affected by an early phase of CKD or are really healthy but have, in addition to the increased creatinine concentration typical of this breed, a physiologically high concentration of SDMA or any other condition that induces a transient decrease of GFR.

In any case, the presence of a higher, although non significant, proportion of cats with SDMA higher than the reference intervals induced a significant difference between Holy Birmans and other breeds of cats in terms of SDMA concentration, as for creatinine, for which, however, the overlap between data of the two breed groups was less pronounced. Moreover, the transference method did not allow validating the pre-existing reference interval. In the generation of the new reference interval a single outlier was found, but it was retained in the dataset since low values of SDMA do not have a clinical significance. However, the breed-specific reference interval generated in this study for Holy Birman cats was largely overlapping with the pre-existing one, and despite the upper reference limit was higher than that recommended for the whole population of cats, its huge confidence interval, depending on a single cat with very high SDMA value, suggests that the reference interval of the general feline population may be used in routine practice, taking into account that, occasionally, values higher than this reference interval could be detected.

Taken together, all these results confirm that, independently on the breed, SDMA, that is considered a sensitive marker of CKD, has not an absolute specificity, as reported in previous studies (Hall et al., 2014a), and that increased SDMA may be occasionally found in Holy Birman cats. However, the comparison of the frequency and magnitude of

increased SDMA and increased creatinine in Holy Birman cats, confirms the hypothesis of this study, that SDMA is less frequently and less severely increased in Holy Birman cats than creatinine. The design of this study was not adequate to investigate the mechanisms responsible for this difference. However, the different behavior of the two analytes suggests that the high creatinine concentration of Holy Birman cats does not depend on a decreased glomerular filtration rate, which, based on our knowledge on SDMA, would more likely induce increases of SDMA than of creatinine (Hall et al., 2014a) (Fleck et al., 2003) (Braun et al., 2003). It has been demonstrated that SDMA is less affected by body mass than creatinine (Hall et al., 2014b) (Hall 2015 et al., 2015), and that in other species, physiologically high creatinine concentration are found in breeds with increased muscle mass compared with the other breeds (Zaldívar-López et al., 2011).

However, the muscle mass of Holy Birman cats is not particularly different from those of other cats and it is therefore unlikely that the different behavior of the two analytes depends on the muscle mass. Similarly, the differences between Holy Birman cats and cats of other breeds cannot be explained by the significant difference recorded for age that, if present, would have more likely induced the opposite effect (frequent increases of SDMA in the group of older cats, i.e. cats of other breeds rather than in Holy Birman cats).

Whatever is the mechanism responsible for these differences, to be investigated through future studies, on a practical point of view the most important conclusion of this study is that SDMA may be a better marker of the health status of the kidney in Holy Birman cats compared with creatinine. Based on this study, SDMA could be recommended as a marker of choice for routine investigating renal function in this breed (and possibly in any other breed with physiologically high creatinine levels) and it should be included in any general health screening profile of these cats. The coupled analysis of results of creatinine and SDMA would allow to not overdiagnose CKD in Holy Birman cats and to perform additional clinical and laboratory investigations only in those cats on which both these analytes are outside the breed specific reference intervals or largely outside the reference interval of the general feline population.

Conclusion

This study supports the idea that SDMA may be less frequently elevated than creatinine in apparently healthy Birman cats when non-breed-specific laboratory RIs are used. On one hand, this study confirms the importance of using a breed-specific RI for investigating renal function in Birman cats; on the other hand, SDMA could be recommended as a marker of choice for routine investigation of renal function in this breed (and possibly in other breeds with physiologically high creatinine levels) and it should be included in any general health screening profile of these cats. The analysis of both creatinine and SDMA would help to prevent the over-diagnosis of CKD or errors in staging renal disease in Birman cats. It would also help to limit further clinical and laboratory investigations in Birman cats to only those that have creatinine concentrations outside of the breed-specific RIs or SDMA concentrations that are largely outside of the RI of the general feline population

4. Serum and urinary biomarker in cats

General materials and methods: patients enrolment and sample collection

Client owned cats were prospectively enrolled between November 2014 and September 2017. All cats were presented for routine health screening at the Internal Medicine unit of the Veterinary Teaching Hospital (Department of Veterinary Medicine, University of Milan)
Informed consent was signed by the owners and, according to the ethical committee statements of the University of Milan (number 2/2016), biological samples collected in this setting could be used also for research purposes. The study populations included both cats at risk to develop CKD and cats already affected by CKD.

Two populations of cats were included in this study:
- healthy cats older than 8 years of age and healthy cats belonging to breeds potentially affected by familiar CKD (i.e. Abyssinian, Persian, Maine Coon) at any age.
- cats affected by CKD at any age and breed; CKD was diagnosed and staged according to criteria listed in Table 1, Table 4 and Table 5.

Exclusion criteria were the following: cats affected by UTI, pre-renal or post-renal proteinuria, cardiovascular diseases, malignant neoplasia, hyperthyroidism or other endocrinopathies; treatments with drugs affecting blood pressure (such as glucorticoids, ACEi, calcium channel blockers). In case of UTI, the patient (or the urinary sample) was included after the resolution of infection.

To identify patients that fulfilled inclusion criteria, a complete physical examination, the measurement of SBP and collection of boold and urine samples were performed at each cat at the visit,. SBP was measured after a period of acclimatization by the Doppler technique (Minidop ES-100VX, Hadeco, Kanagawa, Japan) according to published guidelines. (Brown et al., 2007; Taylor et al., 2017) Specifically, the mean values of three consecutive and consistent (<20% variability) values were recorded.
In case of non-collaborative cats or evidences of stress before the measurement of SBP, a further clinical visit (within the following 7 days) was proposed to the owner in order to re-evaluated only SBP and avoid any other stressful clinical procedure.

SBP was also evaluated 7 days later in case of abnormal high values, in order to exclude the white-coat hypertension (Belew et al., 1999), maintaining the same method of measurement (i.e. position, body site and cuff)

Blood samples were collected by venepuncture from cephalic or jugular vein after 12 hours fasting. 1 mL of blood was placed in EDTA and the remaining volume (on average 2,5 mL) was placed in a plain tube to obtain serum.

CBC was performed with aumated cell counter (Sysmex XT 2000 iV, Sysmex® Co, Kobe, Japan). A biochemistry panel (including Sodium, Phosphorous, Calcium, Potassium, Urea, Creatinine, Glucose, Total Proteins, Cholesterol, Triglycerides, ALT, ALP, GGT) was performed with an automated biochemical analyser (Cobas Mira, Roche® Diagnostic, Basel, Switzerland). Serum electrophoresis was performed with Sebia Hydrasis (Sebia Italia Srl, Bagno a Ripoli, Firenze, Italia). Fiv/Felv test was performed with rapid immuno-chromatographic snap test (Feline FIV/FeLV combo test, IDEXX laboratories, IDEXX Europe B.V., Hoofddorp, The Netherlands) whenever not previously reported and epidemiologic data suggested the execution.

At least 7 mL of urine were collected by ultrasonographic-guided cystocentesis. In cats with CKD or in cats with signs of UTI, an aliquot of urine (1 mL) was sent to the microbiologist for bacterial culture.

A complete urinalysis was performed within 4 hour from collection with the following test: macroscopic physical examination of urine; chemical analysis with reagent strips (Combur 10 test, Roche diagnostics, Risch-Rotkreuz, Switzerland); USG quantification with a portable refractometer (Sper scientific, Scottsdale, USA); urinary sediment evaluation according as previously described (Rossi et al., 2012); and UPC quantification by measuring urinary total proteins with pyrogallol red molybdate (Urine proteins, Sentinel diagnostics, Milan, Italy) and urinary creatinine (Creatinina, Real-Time Diagnostics, Viterbo, Italy) methods applied to Cobas Mira analyser (Cobas Mira, Roche® Diagnostic, Basel, Switzerland)

Other exams (e.g. abdominal ultrasonography) were performed case by case depending on the clinical and laboratory status.

At each visit, cats affected by CKD were staged according to IRIS staging (Elliott and Watson, 2016). All patients were monitored over a 18 months period and cats at risk to develop CKD (IRIS stage 0) and at IRIS stage 1 were checked every 6 months whereas

cats at stage 2-4 were checked every 3 month, unless worsening of the clinical condition will recommend additional sampling to assess their health status.

In order to perform the study (biomarker studies) described below, at first visit and at each checkpoint, aliquots of serum (100 µL) were stored at -20°C in order to measure the serum concentration of big Endothelin-1 (big-ET1, homocysteine and aldosterone. Aliquots of urine supernatant (300 µL) were stored to measure the concentration of alpha-1 microglobulin and big-ET1, and to performer the urinary protein electrophoresis with sodium dodecyl sulfate-agarose gel electrophoresis (SDS-AGE).
Since the volume of serum and urine were not sufficient for all the biomarker studies, not all samples were used in each study.
However, in the studies described below only the samples included in this prospective collection were used.

General results

Forty-eight cats were included in this prospective study which from which it was possible to collect 99 serum and urinary samples.
At the time of inclusion 19 (39.6 %) were staged as At risk, 6 (12.6 %) in stage 1, 16 (45.2%) at stage 2, 4 (8.3) at stage 3 and 3 (6.3 %) at stage 4.
Among the 29 cats with CKD (Table 10), 13 were followed over time (5 for three months, 5 for twelve months, one for fifteen months and 2 for eighteen months). Three cats progressed toward higher stage of CKD (one from stage 1 to stage 2, one from stage 2 to stage 3 and one from stage 3 to stage 4) and 10 remained in the same IRIS stage.
Among the 19 cats at risk (Table 11), 17 cats had follow-up available (5 for six months, 5 for twelve months 2 for fifteen months and 5 for eighteen months). Eight patient progressed toward CKD (4 to stage 1 and 4 to stage 2) and 9 remained stable during the time monitored.
Median and interquartile range of the main CBC and biochemistry results obtained from the enrolled cats are shown in Table 12.

Table 10 Cats with CKD included in the biomarker study. Breed, sex, age (months), presence of symptoms related to CKD, duration of the follow-up (months), stage IRIS based on serum creatinine (Stage Crea) with progression or stability of CKD with stage reached, sub-stages IRIS based on systolic blood pressure (Stage SBP) and sub-stages IRIS based on urinary protein-to-creatinine ratio (Stage UPC) are shown.

N°	Breed	Sex	Age	Symptoms	Follow-up	Stage (progression)	stage SBP	Stage UPC
1	Short air	MC	114	H	18	1 (s)	AP0 (s)	NP
2	Short air	FC	181	S	nf	1	n	NP
3	Short air	MC	75	H	nf	1	AP3	NP
4	Short air	MC	134	S	3	1(P)	AP0	n
5	Exotic S	FC	138	H	nf	1	AP0	BP
6	Persian	MC	174	S	12	1 (s)	AP2	BP
7	Short air	MC	136	H	12	2 (s)	n	NP
8	Short air	FC	226	S	nf	2	AP3	NP
9	Siberian	FC	84	S	12	2 (s)	n	BP
10	Siberian	FC	72	H	15	2(P)	AP0	NP
11	Siamese	FC	128	H	nf	2	n	NP
12	Short air	MC	173	S	nf	2	AP0	NP
13	Norwegian	FC	106	H	12	2 (s)	AP0 (P)	NP (P)
14	Siamese	MC	183	S	nf	2	n	BP
15	Short air	MC	180	S	nf	2	AP2	NP
16	Short air	FC	120	S	3	2 (s)	AP0** (s)	P
17	Short air	FC	144	S	3	2 (s)	AP0	NP
18	Short air	MC	190	S	nf	2	n	NP
19	Short air	FC	176	S	12	2 (s)	AP0 (s)	NP
20	Chartreux	FC	80	S	nf	2	AP0	NP
21	Birman	MC	183	S	nf	2	AP1	n
22	Short air	MC	87	S	3	2 (s)	AP0 (P)	P
23	Short air	FC	171	S	nf	3	AP2	NP
24	Short air	MC	195	S	3	3 (P)	AP2 (s)	P
25	Short air	MC	224	S	nf	3	n	P
26	Short air	FC	200	S	18	3 (s)	AP0 (s)	NP
27	Short air	M	210	S	nf	4	AP0	P
28	Norwegian	FC	98	S	nf	4	AP0	BP
29	Short air	FC	121	S	nf	4	n	P

MC: male castrated, M: intact male; FC: female castrated; S: sintomatic, H: healthy; nf: no follow-up P: proteinuric, NP: non proteinuric (UPC<0.2), BP: borderline proteinuric (UPC=0.2-0.4); P: proteinuric (UPC>0.4); (s): stable. (p): progressive, AP0: SBP<150 mmHg; AP1: SBP=150-160 mmHg; AP2: SBP=160-180 mmHg; AP3: SBP>180 mmHg; n: not available. ** : presence of white-coat hypertension

Table 11 Cats at risk to develop CKD included in the biomarker study. Breed, sex, age (months), duration of the follow-up (months), stability or progression toward CKD (with stage reached), sub-stages IRIS based on systolic blood pressure (Stage SBP) and sub-stages IRIS based based on proteinuri (UPC, urinary protein-to-creatinine) are shown.

N°	Breed	Sex	Age	Follow-up	Progression (stage)	Stage SBP	Stage UPC
1	Short air	FC	169	18	S	AP0	BP
2	Short air	FC	103	12	P (1)	AP0	NP
3	Short air	FC	88	6	P (2)	n	NP
4	Short air	FC	133	12	P (2)	AP0	BP
5	Short air	FC	137	nf	nf	n	nn
6	Short air	FC	108	6	P (1)	AP2	BP
7	Europeo	FC	122	18	P (1)	AP2	NP
8	Exotic S.	FC	85	18	P (1)	AP0 (wch)	NP
9	Short air	FC	131	6	S	AP0	BP
10	Short air	MC	131	6	S	AP0	BP
11	Short air	MC	162	6	P (2)	AP0	NP
12	Maine Coon	MC	71	12	S	AP0	NP
13	Persian	MC	67	18	P (2)	n	NP
14	Short air	MC	167	12	S	n	BP
15	Short air	FC	101	15	S	AP0	NP
16	Short air	MC	164	15	S	n	NP
17	Short air	M	108	12	S	AP0	NP
18	Short air	MC	142	18	S	nn	NP
19	Short air	FC	133	nf	nf	nn	NP

MC: male castrated, M: intact male; FC: female castrated; wch: white-coat hypertension; nf: no follow-up NP: non proteinuric (UPC<0.2), BP: borderline proteinuric (UPC=0.2-0.4); S: stable. P: progressive, AP0: SBP<150 mmHg; AP2: SBP=160-180 mmHg.

Table 1 Descriptive statistics (median, minimum and maximun values) of the complete celle count (CBC), serum biochemistry parameters, urinary protein-to creatinine ratio and urinary specific gravity obtained from the cats included in this study. Results are grouped according to the health status (at risk vs CKD) and according to the time of collection (samples collected at time of enrolment in the study vs samples collected at any time during the follow-up).

	At risk (inclusion)				CKD (inclusion)				At risk (all samples)				CKD (all samples)			
	N°	Median	Min	Max	N°	Median	Min	Max	N°	Median	Min	Max	N°	Median	Min	Max
Age (months)	19	131	67	169	29	144	72	226	36	133	67	186	63	138	72	228
SBP (mmHg)	12	130	120	170	21	140	105	230	26	130	100	200	43	140	105	230
Creatinine*	19	1,29	0,79	1,64	29	1,97	1,22	10,93	35	1,28	0,78	1,64	62	1,95	0,89	10,93
SDMA	15	12	8	15	17	16	8	25	27	12	7	15	32	16	8	74
Urea*	19	60	33	101	28	81,5	50	281	35	60	30	107	59	75	6,2	281
Sodium$	16	153	141	157	26	151,8	129	163	32	152,5	140	157	57	151,6	129	163
Potassium$	17	4	3	4,8	27	4,2	2,7	4,9	33	4,1	3	4,8	58	4,2	2,7	5
Calcium*	17	10,5	8,9	12	27	10,6	7,2	13	32	10,05	8,8	12	58	10,65	7,2	13,6
Phosphorous*	18	4,18	2,86	5,35	27	3,64	2,7	26,6	33	4,22	2,86	5,49	57	3,94	2,69	26,6
Glucose*	19	114	72	157	28	115	81	281	35	117	72	172	60	113	73	281
Cholesterol*	17	175	64	332	21	179	56	366	33	174	64	332	52	183	56	366
Triglyceride*	17	36	21	142	19	44	13	99	33	36	21	142	47	40	13	342
ALT#	19	46	31	115	28	53	25	251	35	47	29	229	60	52,5	25	251
ALP#	19	55	26	217	28	44	11	113	35	54	24	1176	59	46	11	189
GGT#	19	1	0	6	28	0	0	4	35	1	0	6	60	0	0	4
Total Protein*	19	7,54	6,59	8,7	28	7,125	5,94	9,26	35	7,28	5,65	8,7	60	7,115	5,94	9,26
WBC (x10^3/µL)	19	8,13	3,06	14,23	27	8,2	3,21	23,56	35	8,13	3,06	14,91	58	8,265	3,21	23,56
RBC (x10^6/µL)	19	9,55	7,9	11,89	27	8,2	4,45	11,05	35	9,43	7,34	11,89	58	8,115	4,41	11,17
Hb (g/dL)	19	13,3	9,3	16,9	27	11,5	5,6	14,8	35	12,9	9,3	16,9	58	10,95	5,6	14,8
Ht	19	40	30	50,9	27	35	18,6	45	35	40	28	50,9	58	34	18,6	45
PLT (x10^3/µL)	19	134	21	555	27	163	1	444	35	145	16	555	58	133	1	444
UPC	18	0,13	0,04	0,3	27	0,16	0,04	5,63	33	0,14	0,01	0,31	58	0,17	0,02	5,63
USG	18	1050	1024	1086	26	1027	1008	1066	33	1050	1022	1086	55	1033	1008	1616

N°: number of samples obtained; SDMA: symmetric dimethylarginine; ALP: alanine transferase; ALP: alkaline phosphatase; GGT: gamma glutamyl tranferase; WBC: white blood cells, RBC: red blood cells; Hb: hemoglobin; Ht: hematocrit; PLT: platelets; UPC: urinary protein-to-creatinine ratio; USG: urine specific gravity. *=mg/dL; £=µg/dL;$=mmol/L; #=U/L;

4.1 Serum and urinary Big Endothelin-1

Introduction and aims

Endothelin-1 (ET-1) is a biological active peptide that in physiologic state it is mainly synthetized by endothelial cells and regulates blood pressure.

In human medicine it was shown that ET-1 is related to many pathologic mechanisms in CKD and its production is increased in hypertension and CKD (Kohan, 2010). Although circulating concentrations of ET-1 do not accurately reflect ET-1 production, since the short-half life and the predominant paracrine and autocrine activity (Dhaun et al., 2006), blood ET levels were higher in people with focal segmental glomeruloslerosis (Chen et al., 2001). Given its potent vasoconstriction activity, the greatest pathophysiological impact of ET-1 in hypertension seems to be relates to its effects on vascular remodeling. ET-1 (in cooperation with Angiotensin II) promotes vascular cell hypertrophy, hyperplasia, inflammatory cell infiltration, and fibrosis (Kohan, 2010). Its deleterious vasocostrictive effects (contributing to the systemic HT) seems to be also enhanced by the impairment of salt and water homeostasis that could occur in CKD (Boesen, 2015).

In the kidney ET-1 is over-expressed in both the glomerulus (by podocytes and mesangial cells) and the tubulocytes in patients with different nephropaties (Chen et al., 2001; Dhaun et al., 2009; Dhaun et al., 2012). In the glomerulus, the enhanced gene transcription occurs with a wide range of stimuli (such as angiotensin II, vasopressin, TNF-alpha, ROS, hypoxia) and it may be viewed as a common renal stress response (Dhaun et al., 2012). In this setting ET-1 cause contraction of podocytes, loss of the slit diaphragm and activation and proliferation of mesangial cells with production of pro-inflammatory cytokines and matrix proteins (Ohta et al., 1991; Dufek et al., 2016); moreover it contributes to reduction of local blood flow (renal blood flow and GFR) by contracting arterioles (Kohan, 2010). On the renal tubular system, the pathological expression of ET-1 induces reabsorption of water and sodium by renal tubular cells (Kohan, 2010). These ultimately contribute to systemic hypertension (Schiffrin, 2005).

Urinary ET-1 correlates with the severity of renal injury (Dhaun et al., 2009) (Chen et al., 2001) and with the degree of proteinuria (Lehrke et al., 2001).

Also in dogs, the increased blood concentration of ET-1, evaluated indirectly by the precursor Big Endothelin-1 (big-ET1), seems to be associated with the severity of CKD and with hypertensionin (Rossi et al., 2013b). Big-ET1 was used instead of ET-1 because

the precursor has longer half-life and the concentration of big-ET1 is proportional to ET-1 (O'Sullivan et al., 2007).

In cats, ET-1 was evaluated in cardiomyopathies, showing that plasma ET-1 was a significant higher in sick cats compared to healthy cats, (Prošek et al., 2004) and in asthma, showing that ET-1 in broncoalveolar lavage fluid was significantly higher in asthmatic cats compared to healthy cats (Sharp et al., 2013).

No information is available regarding ET-1 or big-ET1 in cats with CKD. Therefore, the aim of this study was to gain information about the serum and urinary levels of big-ET1 in cats with CKD, with and without hypertension and proteinuria, using an enzyme-linked immunosorbent assay (ELISA). This is the first work to explore the potential role of big-ET1 as biomarkers of hypertension associated with CKD. Concentrations were measured in cats with staged CKD based on serum creatinine concentration, urinary protein to creatinine (UPC) ratio, and systolic blood pressure, as proposed by the International Renal Interest Society (IRIS).

Material and methods

Measurement of big Endothelin-1

Big-ET1 was measured with a solid phase sandwich ELISA developed for human big endothelin-1 by IBL (IBL international GmbH, Hamburg, Germany). According to the manufacturer's instruction, the precoated (captured) antibodies on the plate was a rabbit IgG against the C-terminal 22–38 amino acid sequence of human Big-ET1. According to a previous study (Biondo et al., 2003) this sequence shows close homology to the feline amino acid sequence. The labeled (secondary) antibody was a rabbit IgG is directed against a different antigenic site (conjugated with horseradish peroxidase, HRP). The declared measurement range was 0.78 – 100 pg/mL. The procedure was performed as recommended by the manufacturer

At the time of analysis, selected sera and supernatants were gently thawed by transferring tubes at 4°C the day before analysis and then at room temperature one hour before analysis. Plates were read using an automatic plate reader (Dasit multiscan, Dasit) at a wavelength of 450 nm.

Selection of the samples

This study was divided in three session of work. In the first session, 10 serum samples and 10 samples of urine were selected. These samples were obtained from different cats at different IRIS stages (at risk and different severity of CKD) and were firstly run in order to subsequently perform a validation study.

In the second session of the study, the validation of the urinary Big-ET1 was performed; the validation of the serum Big-ET1 was not performed since almost all the serum samples in the previous session failed to yield results above the detection limit of the test. Moreover, further 10 serum samples were evaluated at that time.

Finally, in the third session 59 urinary and 10 serum samples were assayed.

In case of urinary tract infection at the time of inclusion and no supernatants available within one month after recovery, the T0 of urinary was considered the following checkpoint.

Method validation

Urinary samples tested in the first session of work were refrigerated overnight and were used the day after to prepare the reference material for the method validation. Specifically, a “high pool” and a “low pool” were prepared by mixing the samples with the highest and the lower Big-ET1 concentration, respectively. The intra-assay variability was determined by measuring the Big-ET1 concentration of the 2 pools 5 times in the same kit. The mean value, the standard deviation (SD) and the coefficient of variation (CV = SD / mean X 100) were calculated using Excel spreadsheet. The linearity under dilution test was performed by serially diluting the “high pool” by a twofold dilution scheme (i.e 1:2, 1:4, 1:8 and 1:16) using distilled water. Dilutions were tested in duplicate. The mean between the two results and the expected values corresponding to each dilution were calculated. Linear regression between expected and observed results was applied. The percentage of recovery of the observed values compared with expected values at each dilution was also calculated as follow: recovery = mean observed / expected X 100.

Statistical analysis

A commercially available software (GraphPad Prism 5.0; GraphPad Software, San Diego, CA, USA) was used for statistical analysis. A P value <0.05 was considered statistically significant. Distribution of variables was assessed by Kolmogorov-Smirnov test.

The urinary Big-ET1-to-creatinine (Big-ET1:UC) ratio was calculated to normalize the Big-ET1 concentration to the urine dilution.

Mann-Whitney U test was used to compare urinary big-ET1 and urinary Big-ET1:UC ratio between samples grouped according to the presence of CKD (At risk vs CKD) on the basis of SBP (<150 mmHg vs >150 mmHg). Mann-Whitney U test was also used to compare urinary big-ET1 and urinary Big-ET1: UC ratio between samples at risk grouped according to the stability or progression toward CKD; progression of CKD was identified when at the following check-point (6 or 12 months after inclusion) the cat was classified in a higher IRIS stage, according to criteria exposed in Table 1.

Kruskal-Wallis test was used to compare urinary big-ET1, urinary big-ET1:UC ratio between samples grouped according to the IRIS staging. Mann-Whitney U test with Bonferroni correction was used to perform multiple comparisons between the single groups.

Median, range, I and IV interquartile of all the groups were calculated; mean and SD were graphically displayed in figures.

Correlations between variables (SBP, glucose, urea, creatinine, sodium, potassium, calcium, phosphor, cholesterol, triglycerides, ALT, ALP, GGT, total proteins, WBC, RBC, HTC, HGB, PLT, USG, urinary protein, urinary creatinine, UPC, age) were evaluated using Spearman's correlation test.

Results

Serum Big-ET1

Seven patients at risk and 12 cats with CKD were included (one cat were tested two time: time zero and after 3 months). Results obtained from the 20 samples were shown in Table 13. One of the 7 cats at risk (14.3%) and 4 cats of the CKD group (30.8%) had detectable Big-ET1. Concentrations were always low, with the exception of a sample in CKD group (15.81 pg/mL, IRIS stage 3).

Preliminary validation in urine

The mean ±SD of the 5 repeated measurements of the "low pool" was 9.10 ±8.29 pg/mL and the corresponding CV was 91.1% (Table 14). The analysis of the values (measurement n° 2) shows the presence of a "mild outlier". This value is higher than 1.3 times the interquartile range (i.e. 12 pg/dL) (Westgard, 2003).

Table 13 Samples included in urinary evaluation of Big-ET1. The number of cat (see also Table 16), time of sampling (months), the breed, the sex, the age (months), the serum bigET-1 (sBigET1) when available, urinary BigET-1 to urinary creatinine ratio (uBigET-1:UC), stage IRIS based on serum creatinine (Stage Crea), sub-stages IRIS based on systolic blood pressure (Stage SBP) and urinary protein-to-creatinine ratio (Stage UPC) and progression toward IRIS stages based on serum creatinine

Cat	sBigET1	IRIS	stage SBP	Stage UPC
2	0	A	AP0	BP
3	0	A	AP0	NP
5	0	2	AP2	NP
6	0	1	AP0	NP
7	1,86	A	AP0	BP
8	4,02	2	nd	BP
9	2,17	3	AP0	NP
10	0	3	AP2	nd
11	0	1	nd	NP
15	0	3	AP2	P
15	0	4	AP2	NP
19	0	A	AP2	NP
26	0	2	AP0	P
27	0	A	AP0	P
30	0	2	AP2	NP
33	0	A	AP0	NP
35	0	A	AP0	NP
41	15,81	3	nn	P
42	0	3	AP0	P
43	2,51	4	AP0	P

nd = not determined; NP = non-proteinic; BP = proteinuria borderline; P = proteinuria; AP0 = <150 mmHg; AP1 = 150-159 mmHg; AP2 = 160-179 mmHg; AP3 => 180 mmHg

Excluding this value, the mean became 5.50 pg/dL, the SD was than considerably lower (2.27 pg/dL) that the previous one and consequently the CV drops to 41.3 %.
The mean ±SD of the 5 repeated measurements of the “high pool” (Table 14) was 56.74 ±5.60 pg/mL and the corresponding CV was 9.8 %.

Table 14 Results of the 5 within-run (intra-assay) repeated measures of the two pools of urine

Measurement	Low pool BigET1 (pg/mL)	High pool BigET1 (pg/mL)
1	6,09	51.99
2	23,50	65.78
3	4,92	55.76
4	8,21	57.83
5	2,76	52.35

Linear regression demonstrated an excellent degree of correlation (R2 =0.97; P = 0.002) for LUD test (Figure 14).

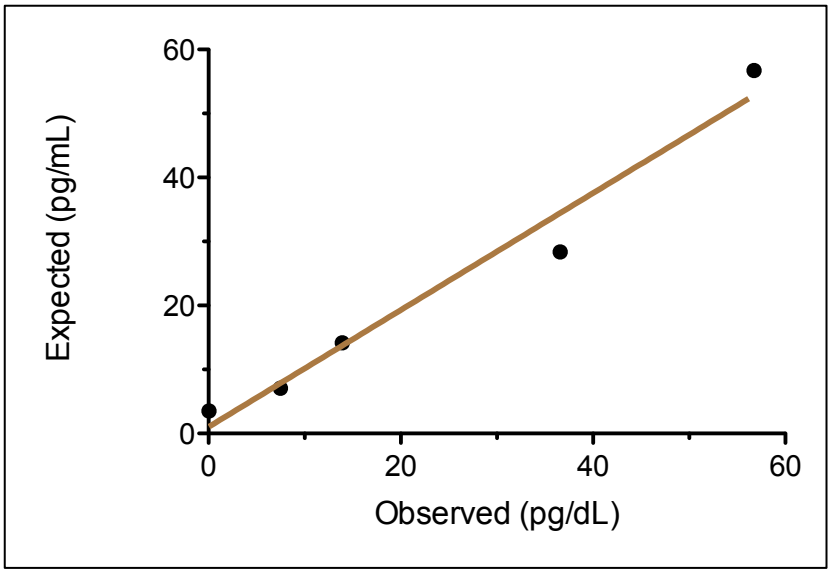


Figure 14 Linearity under dilution (LUD) of “high pool” obtained by mixing two different urine samples. Observed values (in pg/dL) were statistically correlated to the expected value according to a linear model.— intercept: 20.90 (95% confidence interval [CI]: 23.37 to 1.57); slope: 1.05 (95% CI: 0.90–1.21); recovery test— intercept: 22.31 (95% CI: 210.27 to 5.64); slope: 1.17 (95% CI: 0.63–1.71).

The mean percentage of recovery compared with expected values was 83.4%. However, a very low value was found with the 1:16 dilution (Table 15). The expected Big-ET1 concentration was very close to the lower limit of detection of the kit and, as shown above,

also high imprecision can be found at these levels. Excluding this value the mean recovery becomes 110.8%.

Table 15 Results of the linearity under dilution test. The percentage of recovery (O/E) was calculated

Dilution	Observed (pg/mL)	Expected (pg/mL)	O/E (%)
Undiluted	56,74	56,74	
1:2	36,58	28,37	128,9
1:4	13,91	14,19	98,1
1:8	7,47	7,09	105,4
1:16	0,04	3,55	1,3

Urinary Big-endothelin 1—Population of cats

The 69 samples included in urinary evaluation of Big-ET1 belonged to 42 patients, 27 and 3 of which were also sampled other 2 and 3 times in the follow-up, respectively (Table 16).

At the time of inclusion, 18 were classified as At risk and 24 affected by CKD (IRIS 1: 4 cats; IRIS 2: 14 cats; IRIS 3: 4 cats; and IRIS 4: 2 cats).
Mean ±SD of age in At risk group and CKD was 121.9 and 145 months, respectively (range 67-169 and 72-226 months, respectively).
In At risk group were present 7 castrated male, one intact male and 10 neutered female whereas in CDK group 10 castrated male and 14 neutered female.
The majority of cats at risk and cats with CKD were European shortair (15 and 17, respectively)

Table 16 Samples included in urinary evaluation of Big-ET1. The number of cats, the time of sampling (months), the breed, the sex, the age (months), the serum bigET-1 (sBigET1) when available, urinary BigET-1 to urinary creatinine ratio (uBigET-1:UC), stage IRIS based on serum creatinine (Stage Crea), sub-stages IRIS based on systolic blood pressure (Stage SBP) and urinary protein-to-creatinine ratio (Stage UPC) and progression toward IRIS stages based on serum creatinine

Cat	Time	Breed	Sex	Age	sBigET1	uBigET1	uBigET-1 :UC	Stage Crea	stage SBP	Stage UPC	Progression
1	0	Short air	MC	136	Nd	1,07	0,0019	2	Nd	NP	stable
1	12	Short air	MC	147	Nd	2,03	0,0063	2	Nd	NP	nf
2	18	Short air	FC	186	0,00	21,68	0,1325	A	AP0	BP	stable
2	0	Short air	FC	169	Nd	0	0,0000	A	AP0	BP	nf
3	0	Short air	FC	103	0,00	34,29	0,0854	A	AP0	NP	progressive
4	6	Short air	FC	92	Nd	31,83	0,1245	2	AP1	NP	progressive
4	0	Short air	FC	88	Nd	0,95	0,0045	A	Nd	NP	progressive
5	0	Short air	FC	226	0,00	0	0,0000	2	AP2	NP	nf
6	0	Short air	MC	114	Nd	0	0,0000	1	AP0	NP	stable
6	6	Short air	MC	121	Nd	0	0,0000	1	AP0	NP	stable
6	18	Short air	MC	133	0,00	0,99	0,0029	1	AP0	NP	nf
7	0	Short air	FC	133	1,86	6,149	0,0193	A	AP0	BP	progressive
7	12	Short air	FC	143	Nd	0	0,0000	2	Nd	BP	progressive
8	0	Siberian	FC	84	4,02	0	0,0000	2	Nd	BP	stable
9	0	Siberian	FC	72	2,17	14,94	0,0900	2	AP0	NP	progressive
9	15	Siberian	FC	87	Nd	34,64	0,5560	3	AP0	NP	progressive
10	0	Short air	FC	171	0,00	3,07	0,0188	3	AP2	NP	nf
11	0	Short air	FC	108	Nd	0	0,0000	A	AP2	BP	progressive
11	6	Short air	FC	115	0,00	0	0,0000	1	Nd	NP	progressive
12	0	Short air	MC	75	Nd	12,49	0,0506	1	AP2	NP	nf
13	18	Short air	FC	140	Nd	0	0,0000	1	AP2	NP	progressive
13	0	Short air	FC	122	Nd	0	0,0000	A	AP2	NP	progressive
14	0	Siamese	FC	128	Nd	12,22	0,0339	2	Nd	NP	nf
15	3	Short air	MC	199	0,00	8,34	0,3022	4	AP2	NP	progressive
15	0	Short air	MC	195	0,00	0,69	0,0250	3	AP2	P	progressive
16	0	Norwegian	FC	106	Nd	0,73	0,0039	2	AP0	NP	stable
16	12	Norwegian	FC	122	Nd	8,11	0,0466	2	AP3	P	stable
17	0	Short air	MC	87	Nd	0	0,0000	2	AP0	P	stable
18	0	Norwegian	FC	98	Nd	10,02	0,2193	4	AP0	BP	nf
19	0	Exotic S.	FC	85	0,00	16,50	0,0819	A	AP2	NP	progressive
19	18	Exotic S.	FC	102	Nd	0	0,0000	1	AP0	BP	progressive
20	0	Exotic S.	FC	138	Nd	38,91	0,2604	1	AP0	BP	nf
21	0	Siamese	MC	183	Nd	0	0,0000	2	Nd	BP	nf
22	0	Persian	MC	174	Nd	16,54	0,1033	1	AP2	BP	stable
23	0	Short air	MC	180	Nd	3,15	0,0313	2	AP2	NP	nf
24	0	Short air	FC	121	Nd	14,94	0,4640	4	Nd	P	nf
25	3	Short air	FC	123	Nd	15,26	0,0900	2	AP0	P	stable
26	0	Short air	FC	144	Nd	50,67	0,2939	2	AP0	NP	stable
26	3	Short air	FC	147	0,00	14,25	0,1307	2	AP0	P	stable
27	0	Short air	MC	131	0,00	0	0,0000	A	AP0	BP	stable

27	6	Short air	MC	139	Nd	3,85	0,0130	A	AP0	NP	stable
28	0	Short air	FC	131	Nd	0	0,0000	A	AP0	BP	stable
28	6	Short air	FC	139	Nd	2,68	0,0098	A	AP0	NP	stable
29	0	Short air	MC	190	Nd	0	0,0000	2	Nd	NP	nf
30	0	Short air	MC	162	Nd	0	0,0000	A	AP0	NP	progressive
30	6	Short air	MC	169	0,00	0	0,0000	2	AP2	NP	stable
31	12	Short air	FC	189	Nd	0	0,0000	2	Nd	NP	stable
31	0	Short air	FC	176	Nd	0	0,0000	2	AP1	NP	stable
32	0	Chartreux	FC	80	Nd	0,398	0,0015	2	AP0	NP	nf
33	12	Maine Coon	MC	85	Nd	11,92	0,0391	A	AP0	NP	stable
33	0	Maine Coon	MC	71	0,00	4,91	0,0157	A	AP0	NP	stable
34	6	Persian	MC	73	Nd	8,42	0,0210	2	Nd	NP	progressive
34	18	Persian	MC	87	Nd	3,8	0,0112	2	Nd	NP	progressive
34	0	Persian	MC	67	Nd	0	0,0000	A	Nd	NP	progressive
35	0	Short air	MC	167	Nd	1,77	0,0137	A	Nd	BP	stable
35	6	Short air	MC	173	0,00	0	0,0000	A	AP0	NP	stable
35	12	Short air	MC	181	Nd	5,1	0,0546	A	AP0	BP	stable
36	9	Short air	FC	101	Nd	0,35	0,0019	A	AP0	NP	stable
36	0	Short air	FC	111	Nd	0	0,0000	A	AP0	NP	stable
37	0	Short air	MC	164	Nd	0	0,0000	A	Nd	NP	stable
38	6	Short air	M	113	Nd	3,02	0,0090	A	AP0	NP	stable
38	0	Short air	M	108	Nd	0,65	0,0017	A	AP1	NP	stable
39	0	Short air	MC	142	Nd	10,92	0,0763	A	Nd	NP	stable
39	3	Short air	MC	145	Nd	0	0,0000	A	Nd	NP	stable
39	18	Short air	MC	163	Nd	5,92	0,1175	A	AP0	BP	stable
40	0	Short air	FC	133	Nd	0	0,0000	A	AP2	NP	nf
41	0	Short air	MC	224	15,81	13,86	0,3746	3	Nd	P	nf
42	0	Short air	FC	200	Nd	4,99	0,0465	3	AP0	NP	stable
42	12	Short air	FC	213	0,00	14,58	0,2107	3	AP0	P	stable

MC: male castrated; M: male; FC: female castrated; Nd: not determined. NP: non-proteinuric; BP: borderline proteinuric; P: proteinuric; nf: no follow-up; AP0: systolic blood pressure (SBP) <150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP=160-179 mmHg; AP3: SBP >180 mmHg

Urinary Big-endothelin 1—Correlation

Urinary Big-ET1 was significantly negatively correlated with RBC (r =−0.263, P= 0.030), Hb (r=−0.364, P=0.002) and Ht (r=−0.349,P=0.003) (figure 15), and positively correlated with serum creatinine (r=0.241, P=0.046) (figure 16). Also urinary Big-ET1:UC ratio was significantly negatively correlated with RBC (r =−0.367, P= 0.002), Hb (r=−0.451, P=0.000) and Ht (r=−0.442,P=0.000) (figure 15); positive correlation was found with serum creatinine (r=0.321, P=0.007) and urea (r=0.347, P=0.004) (Figure 16)

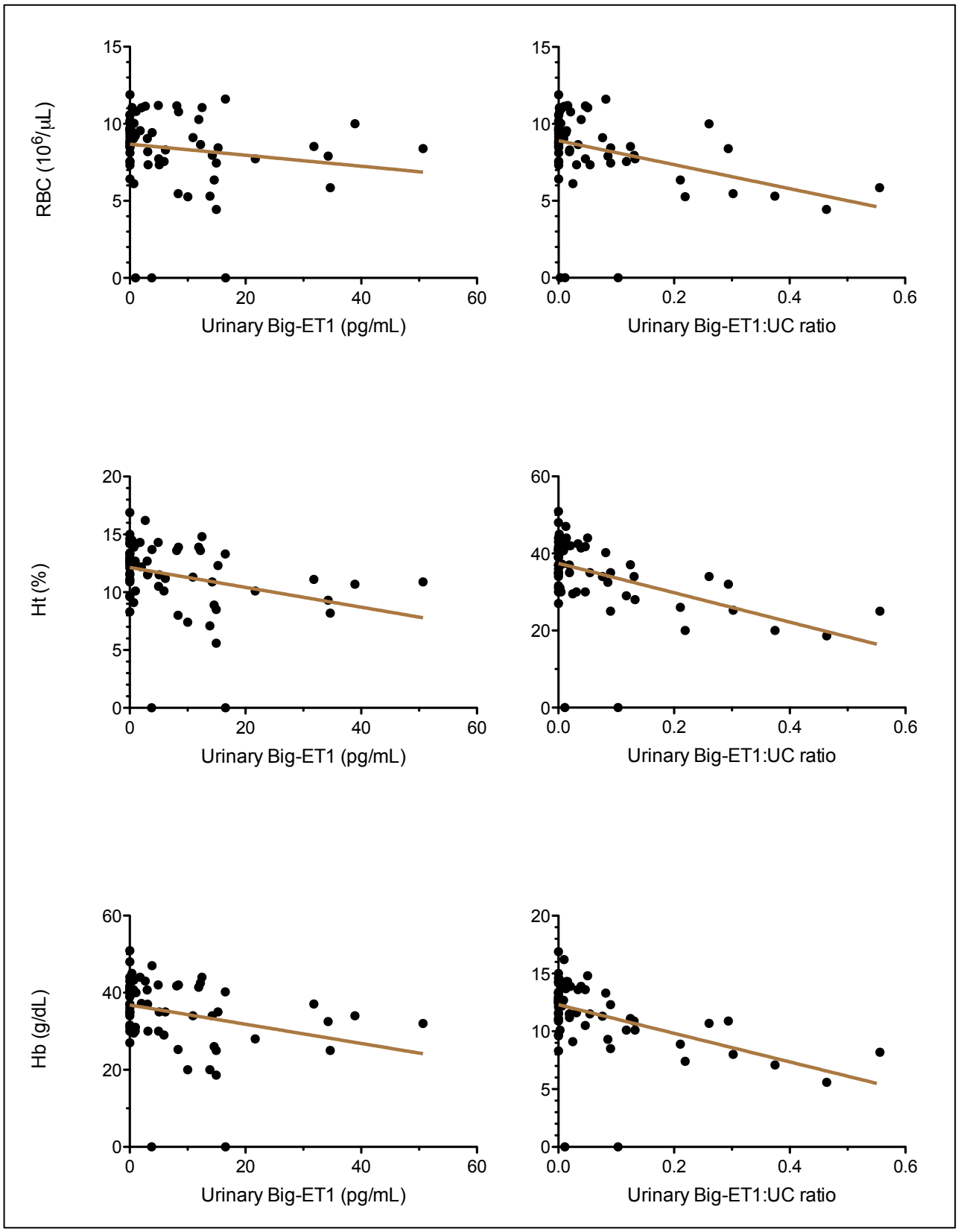


Figure 15 Graphic representation of the correlation between urinary Big-ET1 concentration (on the left) or urinary Big-ET1 to creatinine ratio (on the right) with red blood cells (RBC, top), hematocrit (Ht, middle) and hemoglobin (Hb, bottom). The brown line represent the regression line.

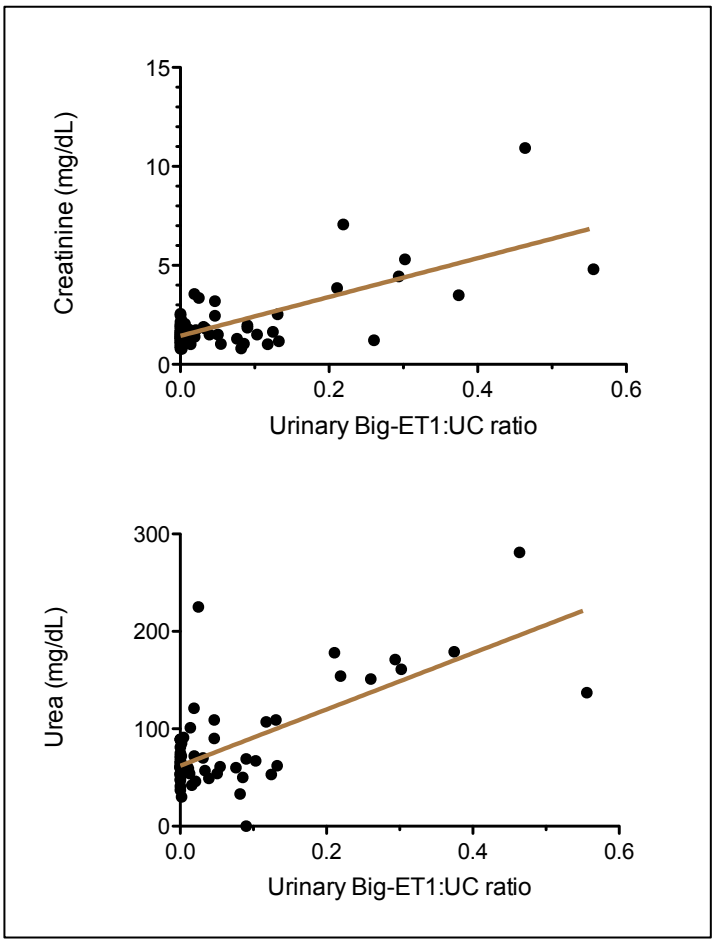


Figure 16 Graphic representation of the correlation between urinary Big-ET1 to creatinine ratio and serum creatinine (top) and urea (bottom). The brown line represent the regression line.

Urinary Big-endothelin 1—Comparison in groups according to serum creatinine

Grouping samples according to the presence of CKD, there was no significantly difference for urinary Big-ET1 concentration (P=0.214) and urinary Big-ET1:UC ratio (P=0.128) between At risk group and CKD group (Figure 17 and Table 17). However, 8 samples in CKD groups had urinary Big-ET1:UC ratio above the higher value obtained in At risk group.

Table 17 Descriptive statistics of the groups At risk and CKD. In brackets are shown the number of cats.

	At risk (n° 28)		CKD (n° 41)	
	BigET1 (pg/mL)	BigET1:UC	BigET1 (pg/mL)	BigET1:UC
Minimum	0,0	0,0	0,0	0,0
25% Percentile	0,0	0,0	0,0	0,0
Median	0,800	0,003	3,070	0,018
75% Percentile	5,715	0,034	14,060	0,114
Maximum	34,29	0,132	50,670	0,556

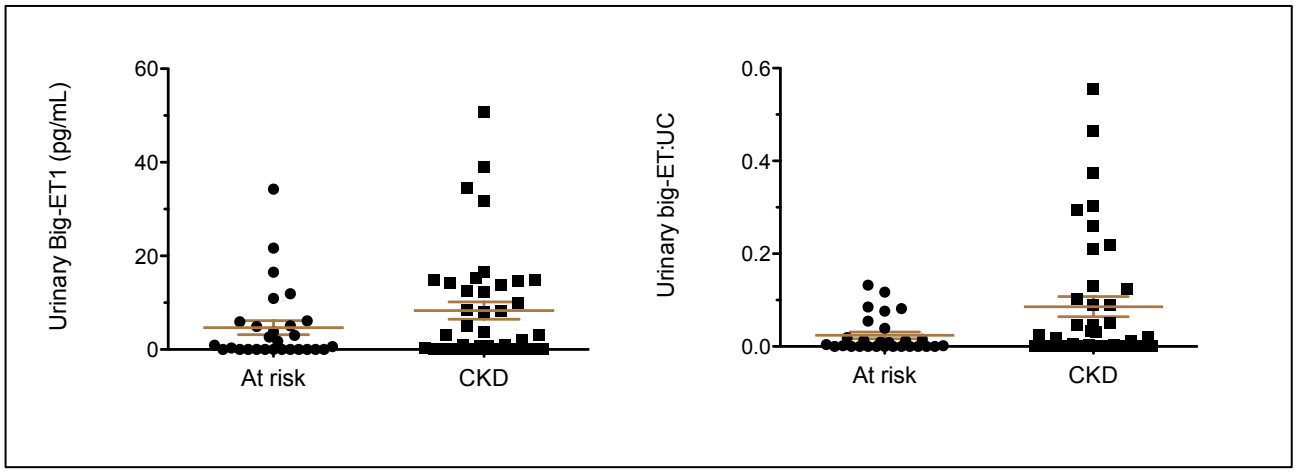


Figure 17 Graphical distribution of urinary Big-ET1 concentration (on the left) or urinary Big-ET1 to creatinine ratio (on the right) between group At risk and CKD

When CKD cats were grouped according to IRIS staging, urinary Big-ET1 was not significant different (P=0.079) between groups, whereas urinary Big-ET1:UC ratio was significantly higher (P=0.001) in IRIS 3-4 group compared to all other groups (Figure 18 and Table 18).

Table 18 Descriptive statistics of the different groups divided according to IRIS Staging. In brackets are shown the number of cats

	At risk (n° 28)		IRIS 1 (n° 9)		IRIS 2 (n° 23)		IRIS 3-4 (n° 9)	
	BigET1 (pg/mL)	BigET1: UC	BigET1 (pg/mL)	BigET1 :UC	BigET1 (pg/mL)	BigET1: UC	BigET1 (pg/mL)	BigET1: UC
Minimum	0,0	0,0	0,0	0,0	0,0	0,0	0,690	0,019
25% Percentile	0,0	0,0	0,0	0,0	0,0	0,0	4,030	0,035
Median	0,800	0,003	0,0	0,0	1,070	0,004	10,020	0,219
75% Percentile	5,715	0,034	14,520	0,077	12,220	0,046	14,760	0,419
Maximum	34,290	0,132	38,910	0,260	50,670	0,294	34,640	0,556

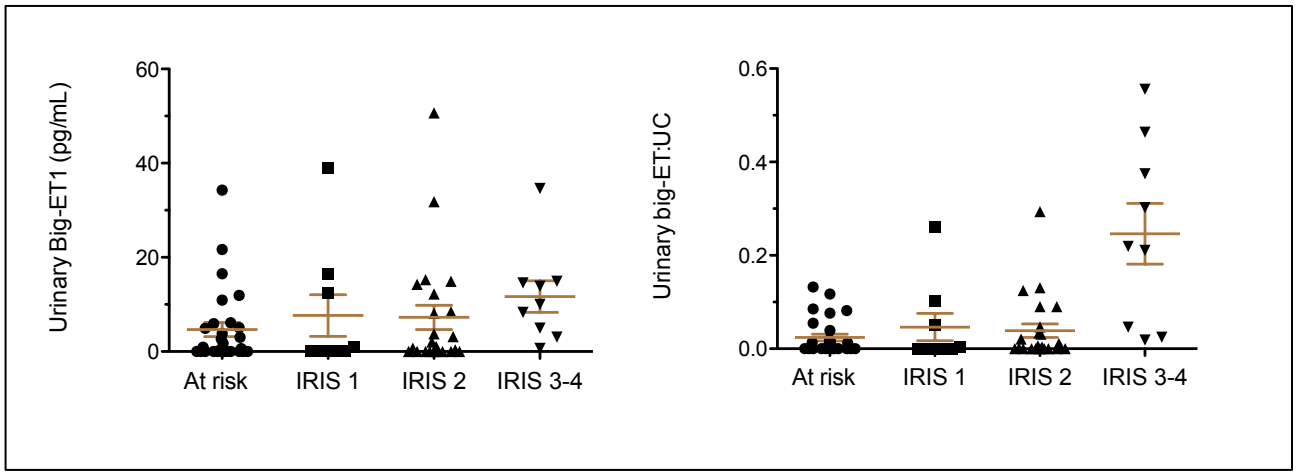


Figure 18 Graphical distribution of urinary Big-ET1 concentration (on the left) or urinary Big-ET1 to creatinine ratio (on the right) in groups groups divided according to IRIS Staging for creatinine.

Both uBig-ET1 (P=0.567) and uBig-ET1:UC ratio (P=0.566) did not significant differed between at risk cats that remained stable and at risk cats that developed CKD (stage IRIS 1 or 2) during the monitored period (Table 19 and Figure 19).

Table 19 Descriptive statistic of cats at risk stable or progressive. In brackets are shown the number of cats

	Stable (n° 9)		Progressive (n° 8)	
	BigET1 (pg/mL)	BigET1:UC	BigET1 (pg/mL)	BigET1:UC
Minimum	0,0	0,0	0,0	0,0
25% Percentile	0,0	0,0	0,0	0,0
Median	0,0	0,0	0,475	0,002
75% Percentile	3,340	0,015	13,910	0,066
Maximum	10,920	0,076	34,290	0,085

0

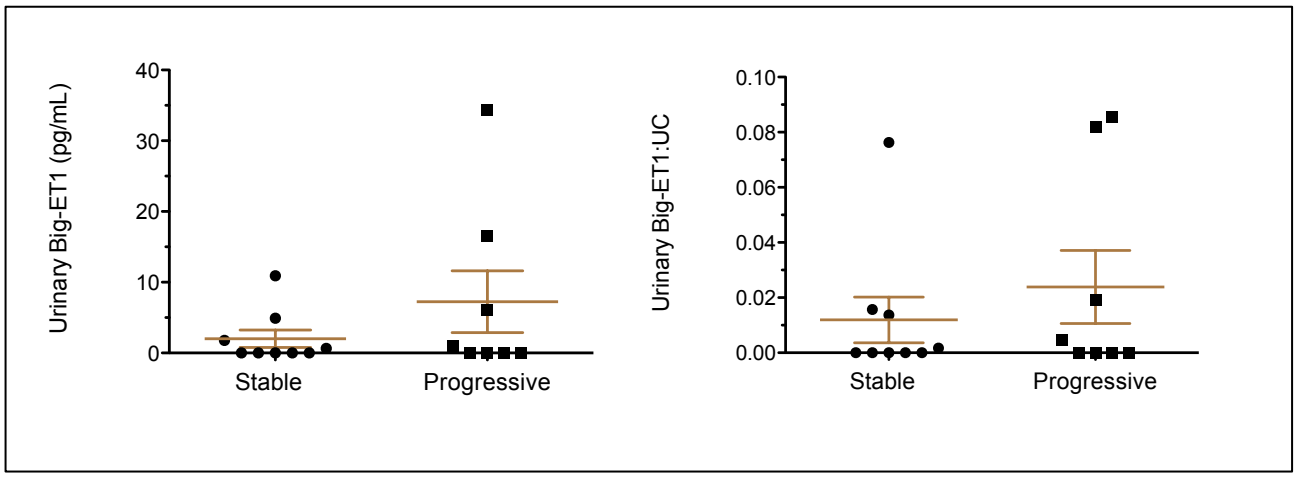


Figure 19 Graphical distribution of urinary Big-ET1 concentration (on the left) or urinary Big-ET1 to creatinine ratio (on the right) in stable and progressive groups

Urinary Big-endothelin 1—Comparison in groups according to SBP

Grouping samples according to SBP, no significant difference was found for uBig-ET1 (P=0.991) and uBig-ET1:UC (P=0.973) between normotensive cats (AP0) and cats with different grade of HT (AP1, AP2 and AP3). In Table 20 and Figure 20 are shown the descriptive statistics and the graphical distribution, respectively.

Table 20 Descriptive statistics of the normotensive (NT) and hypertensive (HT) groups. In brackets are shown the number of cats

	NT (n° 35)		HT (n° 14)	
	BigET1 (pg/mL)	BigET1:UC	BigET1 (pg/mL)	BigET1:UC
Minimum	0,0	0,0	0,0	0,0
25% Percentile	0,0	0,0	0,0	0,0
Median	3,020	0,009	3,110	0,025
75% Percentile	14,250	0,090	13,490	0,087
Maximum	50,670	0,556	31,830	0,302

NT: normotensive; HT: hypertensive

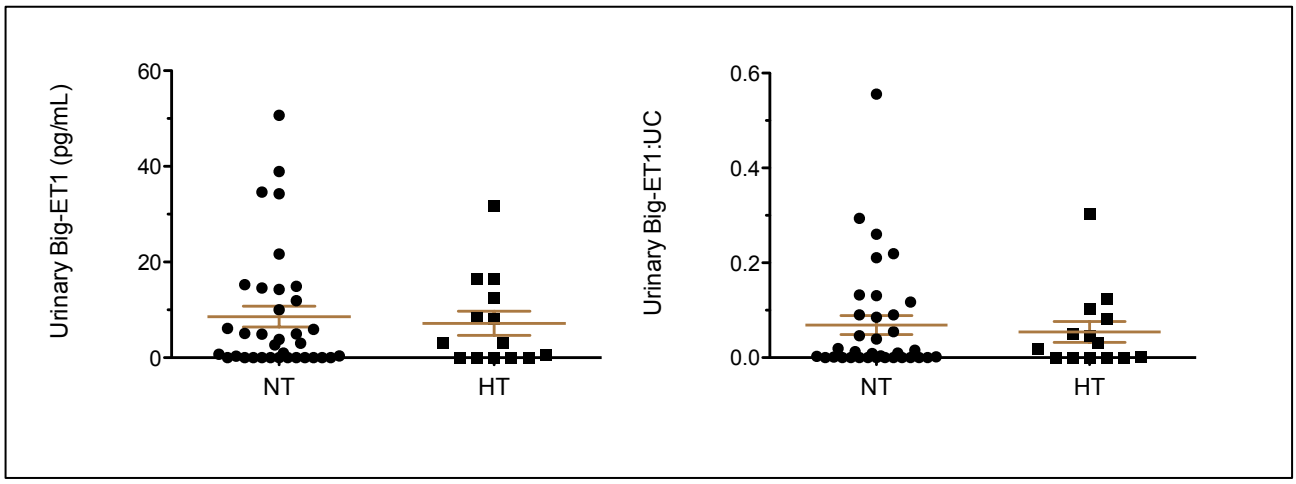


Figure 20 Graphical distribution of urinary Big-ET1 concentration (on the left) or urinary Big-ET1 to creatinine ratio (on the right) in the normotensive (NT) and hypertensive (HT) groups

Given the possible effect of nephropathy on this analysis, each group was further split into two sub-groups according to the presence of CKD. Also in this case, no significant difference was found nor for Big-ET1 (P=0.419) neither for uBig-ET1:UC ratio (P=0.238). (Table 21 and Figure 21)

Table 21 Descriptive statistics of the different groups divided according to systolic blood pressure and presence of CKD. In brackets are shown the number of cats

	At risk NT (n° 17)		At risk HT (n° 13)		CKD NT (n° 18)		CKD HT (n° 10)	
	BigET1 (pg/mL)	BigET1: UC	BigET1 (pg/mL)	BigET1 :UC	BigET1 (pg/mL)	BigET1: UC	BigET1 (pg/mL)	BigET1: UC
Minimum	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
25% Percentile	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Median	3,020	0,009	0,0	0,0	2,990	0,025	5,630	0,039
75% Percentile	6,035	0,047	6,345	0,038	15,02	0,213	13,50	0,108
Maximum	34,290	0,132	17,410	0,081	50,67	0,556	31,83	0,302

NT: normotensive; HT: hypertensive

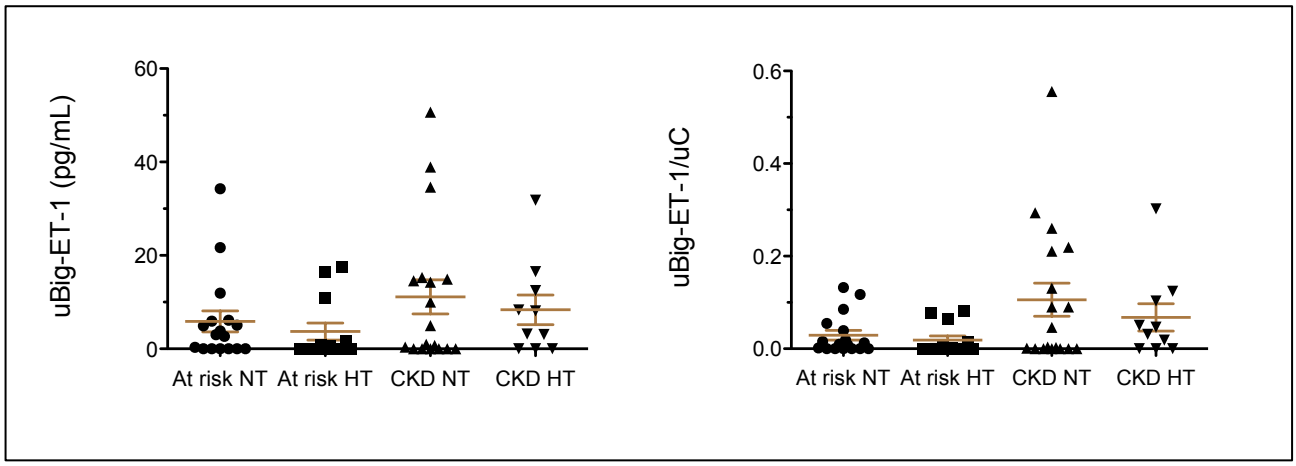


Figure 21 Graphical distribution of urinary Big-ET1 concentration (on the left) or urinary Big-ET1 to creatinine ratio (on the right) in the different groups divided according to systolic blood pressure and presence of CKD

Urinary Big-endothelin 1—Comparison in groups according to proteinuria

Urinary Big-ET1 did not significantly differed (P=0.139) between samples classified according to IRIS sub staging of proteinuria. Conversely, uBig-ET1:UC ratio was 24significantly higher (P=0.026) in P stage compared to NP stage (Figure 22). In Table 22 the descriptive statistic is shown.

Table 22 Descriptive statistics of the different groups divided according to IRIS sub-staging of proteinuria. In brackets are shown the number of cats

	NP (45)		BP (16)		P (8)	
	BigET1 (pg/mL)	BigET1:UC	BigET1 (pg/mL)	BigET1:UC	BigET1 (pg/mL)	BigET1:UC
Minimum	0,0	0,0	0,0	0,0	0,0	0,0
25% Percentile	0,0	0,0	0,0	0,0	2,545	0,0304
Median	0,9900	0,0039	0,8850	0,00685	14,06	0,1104
75% Percentile	8,380	0,0365	9,052	0,1140	14,85	0,3336
Maximum	50,67	0,5560	38,91	0,2604	15,26	0,4640

NP: non-proteinuric; BP: borderline proteinuric; P: proteinuric

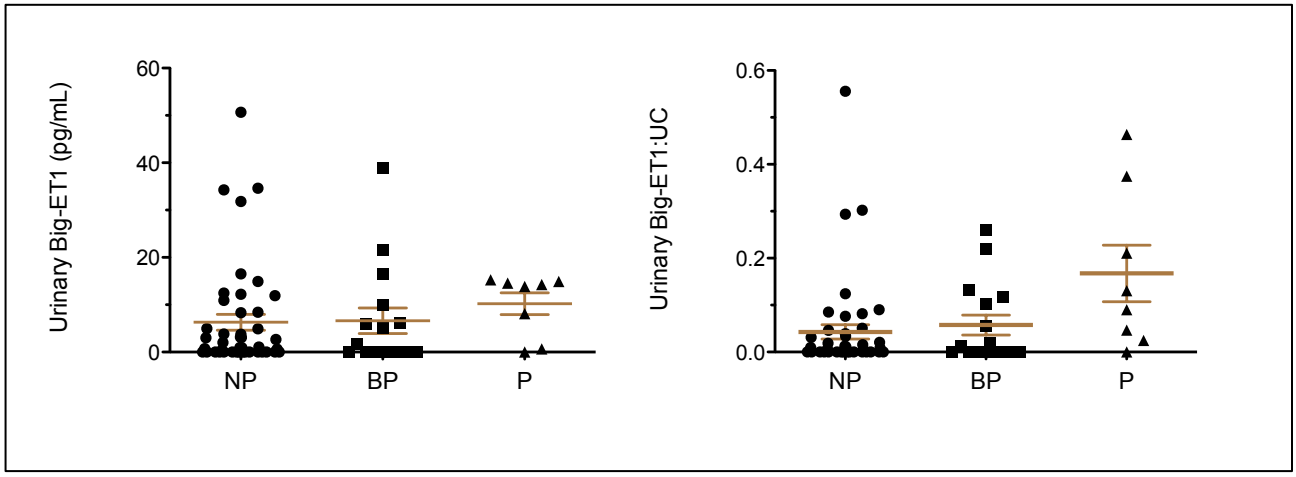


Figure 22 Graphical distribution of urinary Big-ET1 concentration (on the left) or urinary Big-ET1 to creatinine ratio (on the right) in different groups divided according to IRIS sub-staging of proteinuria.

Discussion

To our knowledge, this is the first study to explore this peptide in feline serum and urine. Among the 13 cats with CKD, 5 cat yielded serum Big-ET1 concentration above the limit of detection of the ELISA kit (0.78 pg/mL). It's worth to note that 3 cats were at the late

stages of CKD (IRIS stage 3-4). However other 5 patients were at the same stages but Big-ET1 was not detected.

This absence or low concentration of Big-ET1 in sick cats was unexpected since both human (Miyauchi et al., 2012) and dogs (Rossi et al., 2013b) affected by CKD have high serum Big-ET1. In these species, the concentration tends to be proportional to the severity of the CKD. Specifically, previous studies reported that people at late stages of CKD the mean ±SD of Big-ET1 (measured with a sandwich ELISA) was equal to 17 pg/mL ± 0.2 (Miyauchi et al., 2012), whereas in dogs at IRIS stage 4 of CKD mean ±SD of big-ET1 was 25.26 ±18.64 pg/mL. (Rossi et al., 2013b).

In our few positive cats, only one had serum Big-ET1 with concentration (15.81 pg/mL) similar to values detected in dogs.

It is mandatory to consider these results as preliminary and further studies with higher number of samples are necessary. However, whether these results will be confirmed, different hypothesis could be formulated to explaine the results obtained from serum samples using ELISA kit.

First of all, a possible cause could be the analytical specificity of the ELISA kit. The ELISA kit used in this study is produced by IBL for detection of human big-ET1 and was previously validated in dogs (O'Sullivan et al., 2007) and evaluated in canine CKD by our group of research (Rossi et al., 2013b). In that study the kit was able to detect canine Big-ET1 in serum samples at different concentrations. According to the manufacturer information, the antibodies precoated on the plate of this kit are direct toward the epitope corresponding to the 22-38 sequence of Big-ET1whereas the labeled secondary antibody is directed toward a different epitopes of the polypeptide. In comparison with dog, the 22-38 aminoacidic sequence of the feline Big-ET1is identical. For this reason, considering the homology between canine and feline sequence in that position and the fact that the same kit has been validated successfully for dogs we would expect that the antibody on the plate was able to bound Big-ET1 in feline serum.

Conversely the 1-21 feline sequence differs for an amino acid, as reported by Biondo and colleague (Biondo et al., 2003).

Given this theoretical mild difference in sequence and in turn in structure, and, as described below, our results in urine that are consistent with successful detection of Big-ET1 by the kit, it seems unlikely that the scarce detection of this biomarker in feline serum is due to this specific ELISA kit. However, these evaluations are not sufficient to exclude that the different sequence in cats could reduce the affinity between of the human

antibodies and the feline Big-ET1 and a complete analytical validation of the kit for feline serum are warranted.

To overcome this issue, further investigations using an other ELISA kit or using different assays with higher analytical sensitivity and specificity such as western blot or chromatography are warranted. Moreover, the interaction between of the human labelled antibodies and the feline Big-ET1 could require more time, since the incubation times indicated by the manufacture is ideal for human Big-ET1. Therefore it could be interesting to adjust the protocol performing different incubation periods and temperatures of the labelled antibodies.

Beyond these possible intrinsic problems of the kit, an other possible explanation could be the peculiar metabolism of Big-ET1 in cats. It's known that Big-ET and ET have autocrine and paracrine nature but in other species such as human and dogs it achieve sufficient blood concentration to be detected in serum. No specific information is available about the physiology of this peptide in cats, therefore it could be possible that concentration of Big-ET1 is physiologically low, it could have different half-life and it could be normally degraded with higher rate in blood compared to other species. In this case, the ELISA used in this study would be not enough sensitive to detect big ET in blood and different more analytical sensitive methods would be needed

A further possibility is that only hypertensive patients have detectable Big-ET1. In humans and dogs, Big-ET1 was shown to be correlated to HT (Rossi et al., 2013b). Therefore it could be possible that only cats with high SBP have increased serum Big-ET1 and ET-1. However, the 6 patients with HT were all negative. This hypothesis needs further evaluation with inclusion of more cats with high SBP.

Finally, a last hypothesis could be the presence of other molecules in blood that could interfere with the antibody binding. However also this aspect might be investigated more deeply with proper validation studies because at the moment there is no evidence in literature that support this idea.

Conversely to serum, both validation tests and samples yielded interesting results.

Concerning validation, the pool of samples with high Big-ET1 concentration yielded a CV slightly higher than the limit considered acceptable (5%) for the most common biochemical analytes (Ricós et al., 1999) but widely within the limits considered acceptable for ELISA kits.

The assay was more imprecise with the pool with lower concentrations. This result was not unexpected since it is already known that when the concentration of an analyte in a

sample is low, the slight shift of a measurement from the mean value determines a more profound change of CV compared to a sample with higher concentration (Westgard, 2003). This result suggests that variability between samples at this concentration should be interpreted with caution. Moreover, LUD test showed acceptable recovery, highlighting no or slight matrix effect on the assay.

However, a western blot analysis to confirm the cross-reactivity and a more complete validation following the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2010) are advisable.

In this study we evaluated Big-ET1 instead of ET-1 since it was demonstrated that the former have longer half-life in blood compared to the latter and their concentrations are proportional (O'Sullivanet al., 2007). No information is available about the metabolic pathways of these peptides in urine and further studies are required to confirm that the tight proportionality occur also in this biological sample.

However, assuming that urinary Big-ET1 and ET1 are related as demonstrated in serum, the increase of this biomarker at late stage of CKD and the significant (although weak) correlation with serum creatinine found in this study are in accordance to previous results in humans and rats (Chen et al., 2001; Dhaun et al., 2009).

In a study in people affected by lupus nephritis, urinary ET-1 production increased as renal function declined and was correlated to the renal inflammatory disease activity (Dhaun et al., 2009). In an other study in rats affected by focal segmental glomerulosclerosis, urinary ET-1 excretion was significantly higher in affected animals compared to controls (Chen et al., 2001). ET-1 promotes cell proliferation, hypertrophy, inflammation and extracellular matrix accumulation (Kohan et al., 2014). Since also feline CKD is characterized by chronic inflammation that affects mainly the tubular-interstitium of the kidney (Chakrabarti et al., 2013; Brown et al., 2016), it could be speculated that urinary Big-ET1 in cats could reflect the inflammatory activity of the kidney as demonstrated in human.

Nine cats of the 32 (39.1%) at Stage 2 yielded results under the limit of detection of the method. This result could suggest that individual/biological variation of the urinary excretion of big-ET1 could be high, limiting the use of this biomarker for diagnostic and prognostic purposes and could explain the lack of significant difference between groups.

The variability could also be enhanced by the higher urinary dilution in CKD cats (sometimes present also at early stage of the disease) and the low analytical sensitivity of this method compared to the concentration of Big-ET1 detected in this specie.

The possible different inflammatory activity at each stage mentioned above could be an other possible explanation of the variability between groups and the lack of detectable Big-ET1 in samples at IRIS stage 2.

Urinary Big-ET1 was not able to predict the development of CKD in cats at risk. This result is coherent with the lack of significant difference between the lower stage of CKD (At risk vs IRIS 1 vs IRIS 2) and, according to our results, reflect the clinical importance of ET system only at late stage of disease. However, although the study design was not appropriate to evaluate the prognostic value and few samples were included the analysis.

Proteinuric patients had higher Big-ET1 values compare to non proteinuric and borderline proteinuric patients. However, a wide overlap between groups was shown.

In the study of Chen and colleagues cited above, urinary ET-1 was correlated with the amount of proteinuria (Chen et al., 2001) whereas in the study of Dhaun and collaborators on people with lupus nephritis did not (Dhaun et al., 2009).

It's worth to keep in mind that proteinuria in cats frequently occur at late stages of CKD (Syme et al., 2006). Therefore, given this relation, the tendency of urinary Big-ET1 to increase in proteinuric patients could actually be due to the increased severity of kidney injury, as demonstrated above, instead of a direct effect of urinary proteins. Multivariate analysis with higher number of samples is necessary to evaluate this hypothesis.

It is interesting to note that, in a recent study, glomerular lesions in idiopathic CKD (specifically increased glomerular volume) correlated with proteinuria (Chakrabarti et al., 2013). Podocyte injury is the hallmark of glomerular injury and ET-1 was demonstrated to by one important mediator (Daehn et al., 2014). Therefore it could be hypothesized that urinary ET-1 in cats reflect the glomerular injury. Even more, glomerular lesion are mild and variable at all the stage of CKD in cats (Chakrabarti et al., 2013) and markers of podocyte injury in cats with CKD were not associated with the degree of renal dysfunction (Ichii et al., 2011). Hence these data could be further in accordance with the variability of urinary Big-ET1 levels in our population.

However, to confirm all these hypothesis, further studies including cats with a more strict criteria and/or execution of renal biopsy together to Big-ET1 quantification are necessary.

The lack of significant differences between samples with or without HT suggest that SBP is not an important determinant of the urinary big ET level in cats. This is not an surprising result, since in human medicine urinary ET-1 is considered to reflect mainly renal production (Dhaun, 2006) instead of circulating levels because of its autocrine and

paracrine actions. Whether Big-ET1 and ET-1 are mainly associated to intra-glomerular hypertension instead of systemic hypertension need to be evaluated.

4.2 Serum Homocysteine

Introduction and aims

Homocysteine (Hcy) is a thiol-containing amino acid involved in metabolism of S-adenosyl methionine, folate and B vitamins (Ruaux et al., 2001; Stanger et al., 2003; Rossi et al., 2013a). In people, circulating Hcy is strongly correlated to GFR and increases in CKD (Wollesen et al., 1999). It was hypothesized that two mechanisms are responsible of the hyperhomocysteinemia in case of chronic kidney injury: the reduction of renal excretion due to nephron loss and the impaired extra-renal Hcy metabolism due to the toxic effect of retained substances (van Guldener, 2006). Hyperhomocysteinemia in patients affected by CKD is linked to a greater decline of GFR and to CKD-associated cardiovascular complication (Ninomiya et al., 2004; Levi et al., 2014).

I was also demonstrated that Hcy linearly related to both systolic and diastolic blood pressure (Nygard et al., 1995) and it increases in patients with CKD-associated hypertension and/or proteinuria (Stehouwer and van Guldener, 2003). It was suggested that the possible linking mechanism between hyperhomocysteinemia and hypertension could the effect of the former on arterial walls, by destroying elastin fibers, increasing collagen production and stimulating smooth muscle activity (van Guldener et al., 2003). Also, it could reduce the bioavailability of nitric oxide due to auto- oxidation of homocysteine it may lead to the accumulation of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase (van Guldener, 2006). However, since impaired renal function may increase both SBP and Hcy (Long and Nie, 2016), whether the causal relationship between the two exists is still debated. Independently on the mechanism leading to hypertension, in people hyperhomocysteinemia may works as predictor of hypertension (Wang et al., 2014).

Few information is available about Hcy in veterinary medicine. In dogs, Hcy increase with hypocobalaminemia (Heilmann et al., 2016), in cardiac diseases (Rossi et al., 2008) and in severe CKD similarly to people (Rossi et al., 2008;Rossi et al., 2013b). However, no significant increase of Hcy was found in nephropatic patients with hypertension compared to normotensive CKD dogs (Rossi et al., 2013b). No information is available about Hcy concentration in cats affected by CKD with or without HT. Therefore, the aims of this study were to preliminary validate the enzymatic method in this specie, previously validated in human and dogs (Rossi et al., 2008), and to evaluate whether Hcy increase in cats with CKD and whether it is a marker of CKD-associated HT as in other species.

Material and methods

Selection of the samples

Sixty-five samples obtained from 38 cats were included in this study.
Analyses of the samples were divided into two session of work, performed approximately in the middle and at the end of the study period. In the first phase 30 samples were analyzed and the validation study was carried out. In the second session further 35 samples were analyzed.

Measurement of homocysteine

Hcy was determined by spectrophotometry using an automated biochemical analyzer (Cobas Mira, Roche, Basilea, Swiss) and a commercially enzymatic method (Demeditec Diagnostic GMBH, Rendsburg, Germany). At each session of work, the method was calibrated and controlled using the provided materials. Reported lower limit of detection of the method was 1,5 µmol/L and manufacture declare that the method is linear until 50 µmol/L.

Method validation

Samples tested in the first session of work were used the same day to prepare the reference material for the method validation. Specifically, a “high pool” and a “low pool” were prepared by mixing 5 samples with the highest and the lower Hcy concentration, respectively. The intra-assay variability was determined by measuring the Hcy concentration of the 2 pools 20 times in the same run. The mean value, the standard deviation (SD) and the coefficient of variation (CV = SD / mean X 100) were calculated using Excel spreadsheet. The inter-assay variability was determined by measuring the “high pool” in 5 consecutive days, keeping the serum closed in the tube at +4°C.
The linearity under dilution test was performed by serially diluting the “high pool” by a twofold dilution scheme (i.e 1:2, 1:4, 1:8 and 1:16) using distilled water. Dilutions were tested in triplicate. The mean between the two results and the expected values corresponding to each dilution were calculated. Linear regression between expected and observed results was applied. The percentage of recovery of the observed values compared with expected values at each dilution was also calculated as follow: recovery = mean observed / expected X 100.

Statistical analysis

Commercially available software (Analyse-it, Analyse-it software Ltd, Leeds, UK) was used for statistical analysis. A P value <0.05 was considered statistically significant. Distribution of variables was assessed by Kolmogorov-Smirnov test.

Median, range, I and IV interquartile, mean and standard deviation of Hcy for all the groups were calculated.

Mann-Whitney U test was used to compare Hcy between two groups. Kruskal-Wallis test was used to compare Hcy between 3 or more groups, followed by Bonferroni correction to perform multiple comparison between the single groups. Correlations between variables were evaluated using Spearman's correlation test.

Results

Validation

Results obtained from the 20 repeated readings on high and low serum pools are showed in Table 23.

The high pool provided a mean value of 25.82 µmol/L ± (SD= 1.67 µmol/L), corresponding to a 6.46% CV, slightly above 5%, considered acceptable limit of many analytes commonly used in clinical biochemistry (Ricos et al., 1999). However, the analysis of individual readings revealed the presence of a "far outlier" (reading No. 10), with a value greater than 3 times the interquartile range (0.96 µmol/L) (Westgard, 2003). By removing this outlier, the mean value remains almost unchanged (26.14 mmol/L) but decreases the standard deviation (0.81 µmol/L) and consequently the CV, which is 3.11% and drop below the acceptable limit above-mentioned.

Mean ±SD of the low pool was 9.10 ± 0.67 µmol/L for a CV of 7.36%. Even in this case, there is an outlier (reading 2) even if it is a "near outlier" (ie a value less than 1.5 times the interquartile range (0.68 µmol/L) (Westgard, 2003). Then, even removing this outlier, the mean value, (9.17 µmol/L) the standard deviation (0.61 µmol/L) and CV (6.70%) vary slightly, which in this case also remains slightly above the acceptable limit, usually used for clinical biochemistry determinations (Ricos et al., 1999).

The results obtained in the inter-assay repeatability tests carried out by 5 repeated readings of the high concentration Hcy pool are showed in Table 24. These results correspond to a mean value of 23.92 ± 2.77 µmol/L, and a CV of 11.60%

In this case no outliers have been detected.

Table 23 Results of the 20 within-run repeated measurements on high and low pools

Measurement n°	High pool (µmol/L)	Low pool (µmol/L)
1	26.28	9.50
2	26.19	7.82
3	26.00	8.91
4	26.23	9.65
5	25.09	7.97
6	26.65	8.04
7	25.45	8.08
8	27.82	9.50
9	26.65	8.99
10	19.58	8.69
11	25.63	9.79
12	24.95	8.91
13	26.51	9.68
14	24.50	9.43
15	26.47	9.43
16	27.59	9.06
17	26.09	9.98
18	26.65	9.46
19	26.00	9.87
20	26.09	9.35

Table 24 Results of the five between-run repeated measure-ments on high pool

Measurement n°	High pool (µmol/L)
1	26,28
2	26,19
3	26,00
4	26,23
5	25,09

Linearity under dilution test. The results obtained from the dilution of the high concentrated Hcy pool are shown in Table 25.

Table 25 Results from the serial dilution of the pool with high concentration of homocysteine. On each dilution the reading was performed three times

Dilution	1° reading (µmol/L)	2° reading (µmol/L)	3° reading (µmol/L)	Mean (µmol/L)	SD	CV (%)
0	34.36	34.16	nd	34.26	0.14	0.41
1:2	17.80	13.14	16.95	15.96	2.48	15.55
1:4	8.76	9.13	8.59	8.82	0.28	3.13
1:8	4.04	4.20	3.91	4.05	0.15	3.59
1:16	<2.65	<2.65	<2.65	<2.65	ND	ND

SD: standard deviation; CV (%): coefficient of variability

The intra-assay variability was overlapping or, in much of the cases, much lower than that observed in the above-mentioned imprecision tests, with the exception of 1:2 dilution in which the CV was higher.

Regardless of the repeatability, the analyses of data highlight a progressive decrease of the mean by increasing the dilution. When compared to the expected values (Table 26), the observed values yielded mean ±SD recovery rate of 96.98 ±5.36%, with recovery values slightly below the average in 1: 2 and 1: 8 dilutions and higher in 1:4 dilution. Results of the last determination (1:16 dilution) were excluded from this analysis because they were lower than the detection limit of the method.

Table 26 Serial dilutions of high concentration homocysteine pool with relative observed value (mean of three measurements), expected and recovery rate (O/E recovery)

Dilution	Observed value (µmol/L)	Expected value (µmol/L)	O/E recovery (%)
0	34.26	34.26	ND
1:2	15.96	17.13	93.19
1:4	8.82	8.56	103.12
1:8	4.05	4.28	94.63

The linearity of the results obtained after dilution (Figure 23) was excellent (P = 0.001; r^2 = 0.998)

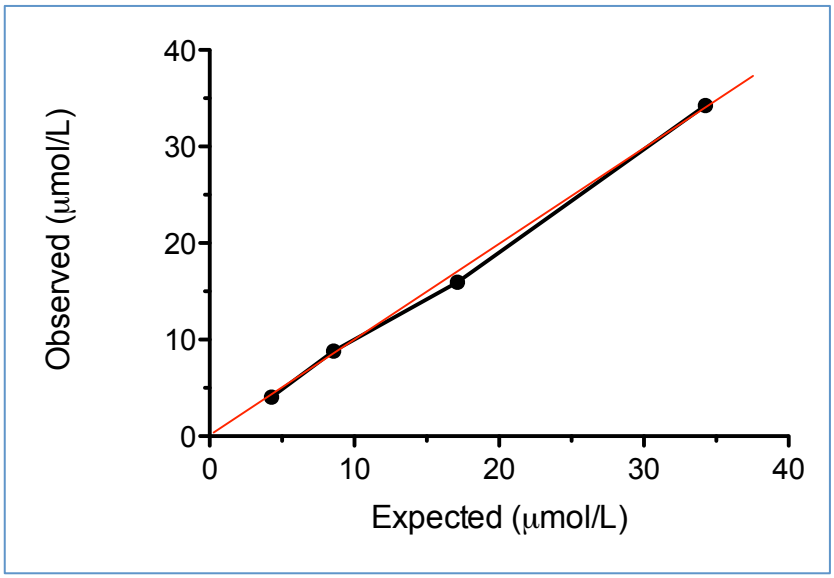


Figure 23 Graphic representation obtained from Linearity tests after dilution (LUD).

Population of cats

The 65 samples included in urinary evaluation of Hcy belonged to 38 patients

At the time of inclusion, 17 were classified as At risk and 21 affected by CKD. Number of affected cats in each of the four IRIS stage (according to serum creatinine) were: IRIS 1 were 5, IRIS 2 were 10, IRIS 3 were 4 and IRIS 4 were 2.

In At risk group were present 7 male and 10 female whereas in CKD group 10 male and 11 female. The majority of cats at risk and cats with CKD were European Short air (14 and 14, respectively). The results obtained in the 29 sample of healthy cats at risk of CKD are listed in Table 27. Samples obtained from cats at risk showed a relative homogeneity of values, with data predominantly distributed between 3.37 and 16.7 µmol/L, with the exception of two samples (numbers 11 and 19), showing particularly high values (30.7 and 40.2 µmol/L). Although both of these cats were clinically healthy at the time of inclusion in this study, the 12-month follow-up after sampling revealed, in one case, the appearance of CKD 6 months after collection, and in the other the appearance of clinical signs and laboratory alterations compatible with hyperthyroidism one year after the sample collection. The mean value obtained in the group at risk was 10.58 ± 7.78 µmol/L (median: 8.44 µmol/L), although this distribution is influenced by the presence of the two outliers described above. Indeed, excluding the outliers, the value corresponds to 8.74 ± 3.48 (median: 7.95 µmol/L).

The results obtained in the 36 nephropatic cats are shown in Table 28.

Table 27 Cats at risk included in the study with collection time, IRIS classification of creatinemia (stage Crea), proteinuria (stage UPC) and systolic blood pressure (stage SBP), and value obtained from the measurement of homocysteine

Sample n°	Cat n°	Time	Stage Crea	Stage UPC	Stage SBP	Hcy (µmol/L)
3	2	T0	A	BP	AP0	5.42
4	2	T9	A	NP	AP0	7.10
5	2	T18	A	BP	AP0	6.11
6	3	T0	A	NP	AP0	9.61
7	4	T0	A	NP	nd	8.49
11	7	T0	A	BP	AP0	40.20
19	11	T0	A	BP	AP2	30.70
23	14	T0	A	NP	AP2	8.86
24	14	T6	A	NP	AP3	4.27
25	14	T12	A	NP	AP3	7.36
34	20	T0	A	NP	AP2	7.95
35	20	T12	A	NP	AP1	7.36
42	25	T0	A	BP	AP0	10.60
43	25	T6	A	NP	AP0	11.40
44	26	T0	A	P	AP0	11.70
45	26	T6	A	NP	AP0	16.70
46	27	T0	A	NP	AP0	14.70
51	29	T0	A	NP	AP0	3.37
52	29	T12	A	NP	AP0	8.44
53	30	T0	A	NP	nd	15.70
54	31	T0	A	BP	nd	13.30
55	31	T6	A	NP	AP0	7.06
56	31	T12	A	BP	AP0	6.69
57	32	T0	A	NP	AP0	4.62
58	33	T0	A	NP	AP1	9.15
59	33	T12	A	nd	AP0	6.29
60	34	T0	A	NP	nd	5.35
61	34	T3	A	NP	nd	11.60
62	35	T0	A	NP	AP2	6.82

Hcy: Homocysteine; T0: collection time executed at time of inclusion; T3: three months from T0; T6: 6 months from T0; T9: 9 months from T0; T12: 12 months from T0; T18: 18 months from T0; A: at risk; nd: not available; NP: non-proteinic; BP: borderline proteinuric; P: proteinuric; AP0: systolic blood pressure (SBP) <150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP=160-179 mmHg; AP3: SBP >180 mmHg

Table 28 Cats affected by CKD included in the study with collection time, IRIS classification of creatinemia (stage Crea), proteinuria (stage UPC) and systolic blood pressure (stage SBP), and value obtained from the measurement of homocysteine

Sample n°	Cat n°	Time	Stage Crea	Stage UPC	Stage SBP	Hcy (µmol/L)
1	1	T0	2	NP	nd	15,10
2	1	T12	2	nd	nd	17,80
8	5	T0	2	NP	AP3	9,47
9	6	T0	1	NP	AP0	12,60
10	6	T12	2	NP	AP0	4,94
12	8	T0	2	BP	nd	16,70
13	8	T12	2	nd	nd	17,10
14	9	T0	2	NP	AP0	7,20
15	9	T3	3	NP	AP0	9,10
16	9	T12	3	NP	AP0	16,40
17	9	T15	3	NP	AP0	13,80
18	10	T0	3	NP	AP2	7,72
20	11	T6	1	NP	nd	38,50
21	12	T0	1	NP	AP3	16,00
22	13	T0	4	P	AP0	23,40
26	14	T18	1	NP	AP2	6,96
27	15	T0	3	P	AP2	15,00
28	15	T3	4	NP	AP2	17,70
29	16	T0	1	nd	AP0	8,62
30	17	T0	2	NP	AP0	11,30
31	17	T12	2	P	AP3	9,98
32	18	T0	4	BP	AP0	15,80
33	19	T0	1	BP	AP0	10,90
36	21	T0	2	NP	AP2	7,82
37	22	T0	1	BP	AP3	10,80
38	22	T12	1	nd	nd	9,24
39	23	T0	2	P	AP0	11,10
40	24	T0	2	NP	AP0	6,07
41	24	T3	2	P	AP0	11,70
47	27	T6	2	NP	AP3	20,10
48	28	T0	2	NP	AP1	12,10
49	28	T3	2	BP	AP0	17,60
50	28	T12	2	NP	nd	16,70
63	36	T0	3	P	nd	21,10
64	37	T0	2	nd	AP1	8,04
65	38	T0	3	NP	AP0	14,80

T0 =collection time executed at time of inclusion; T3 = three months from T0; T6 = 6 months from T0; T9 = 9 months from T0; T12 = 12 months from T0; T18 = 18 months from T0; nd = not available; NP = non-proteinic; BP = proteinuria borderline; P = proteinuria; AP0 = <150 mmHg; AP1 = 150-159 mmHg; AP2 = 160-179 mmHg; AP3 => 180 mmHg

Comparison based on serum creatinine

Descriptive statistics of the Hcy for the At risk and CKD groups are summarized in Table 29 and shown graphically in Figure 24. Hcy between the two group was statistically different (P = 0.002)

Table 29 Descriptive statistics of results obtained in CKD cats and cats at risk. Mean, median and Min-Max of homocysteine values are shown.

Group	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
CKD	36	13.59 (11.27-15.91) ± 6,24	12.35 (9.98-16.00)	4.94-38.50 (9.16-16.70)
At risk	29	10.58 (8.00-13.17) ± 7,767	8,44 (6.82-11.40)	3.37-40.20 (6.56-11.63)

SD = standard deviation; CI = confidence interval

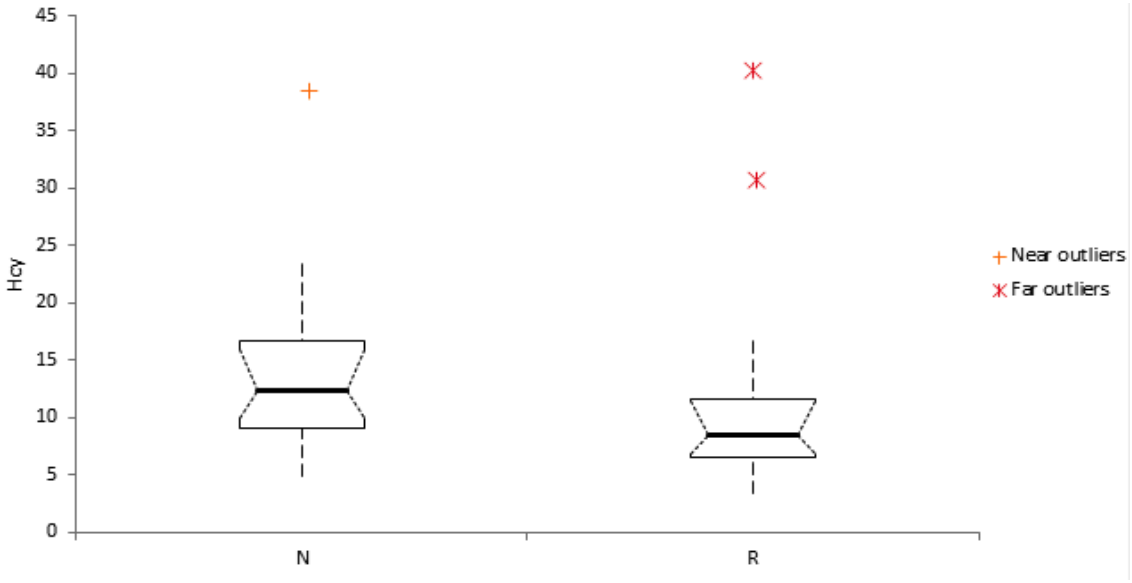


Figure 24 Graphic representation of the comparison between the groups of cats at risk (R) and nephropatic cats (N). Hcy = homocysteine concentration. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

The results obtained by dividing the CKD group in the subgroups according to IRIS staging based on serum creatinine are shown in Table 30 and summarized in Figure 25.

Table 30 Descriptive statistics of results obtained in cats grouped according to IRIS stage. Mean, median and Min-Max of homocysteine values are shown.

Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
A	29	10,58 (7,99-13,18) ±7,77	8,44 (6,82-11,40)	3,37-40,20 (6,56-11,63)
1	8	14,20 (9,26-19,15) ±10,19	10,85 (6,96-38,50)	6,96-38,50 (8,88-14,58)
2	18	12,27 (8,97-15,56) ±4,62	11,50 (8,04-16,70)	4,94-20,10 (8,02-16,73)
3	7	13,99 (8,70-19,27) ±4,50	14,80 (7,72-21,10)	7,72-21,10 (9,88-16,17)
4	3	18,97 (10,90-27,04) ±3,95	17,70 (-)	15,80-23,40 (16,12-22,45)

A = at risk; SD = standard deviation; CI = confidence interval

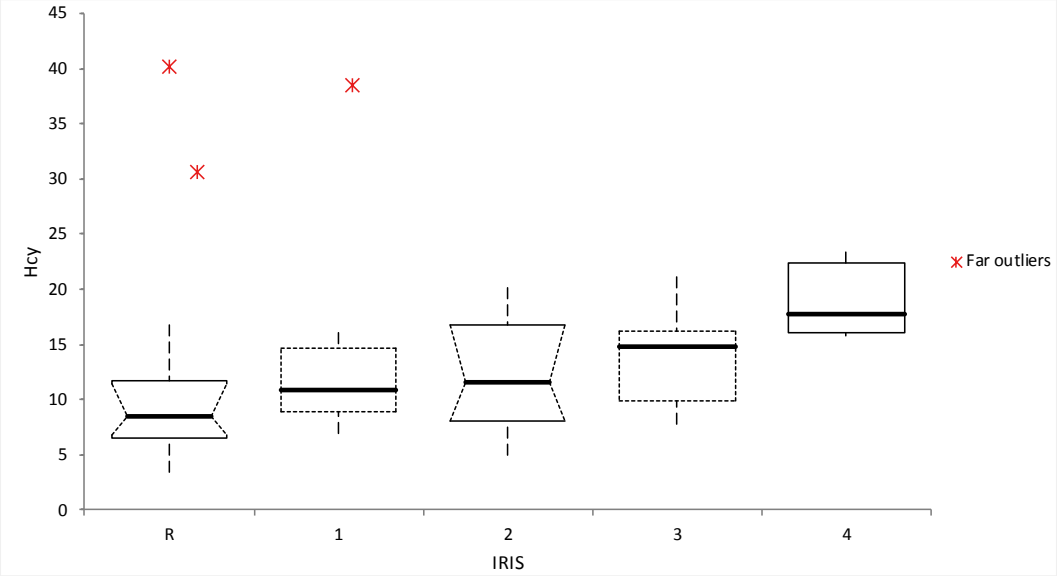


Figure 25 Graphic representation of the comparison between the group of cats at risk (R) and CKD cats divided based on the IRIS stage. Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

This comparison showed a statistical difference between the groups (P = 0.015), but the subsequent post-hoc statistical comparisons between the single groups did not showed significant differences, likely due to the excessive fragmentation of the values, especially for IRIS groups 3 and 4, which were less numerous.

Therefore, we decided to group the IRIS stage 3 and IRIS stage 4 together and to repeat the analysis. The descriptive statistics of the groups (including the new subgroup IRIS 3+4) are summarized in Table 31 and the and graphically represented in Figure 26.

A significant general difference was found (P = 0.013) but, again, there was no significant difference between the single groups despite the pooling of IRIS 3 and 4.

Table 31 Descriptive statistics of results obtained from cats at risk (R) and cats with CKD grouped according to the IRIS staging (IRIS 3 and 4 joined). The mean, median and Min-Max values are reported

Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
R	29	10,58 (7,99-13,18) ± 7,77	8,44 (6,82-11,40)	3,37-40,20 (6,56-11,63)
1	8	14,20 (9,26-19,15) ± 10,19	10,85 (6,96-38,50)	6,96-38,50 (8,88-14,58)
2	18	12,27 (8,97-15,56) ± 4,62	11,50 (8,04-16,70)	4,94-20,10 (8,02-16,73)
3-4	10	15,48 (11,06-19,90) ± 4,77	15,40 (9,10-21,10)	7,72-23,40 (13,41-17,98)

SD = standard deviation; CI = confidence interval

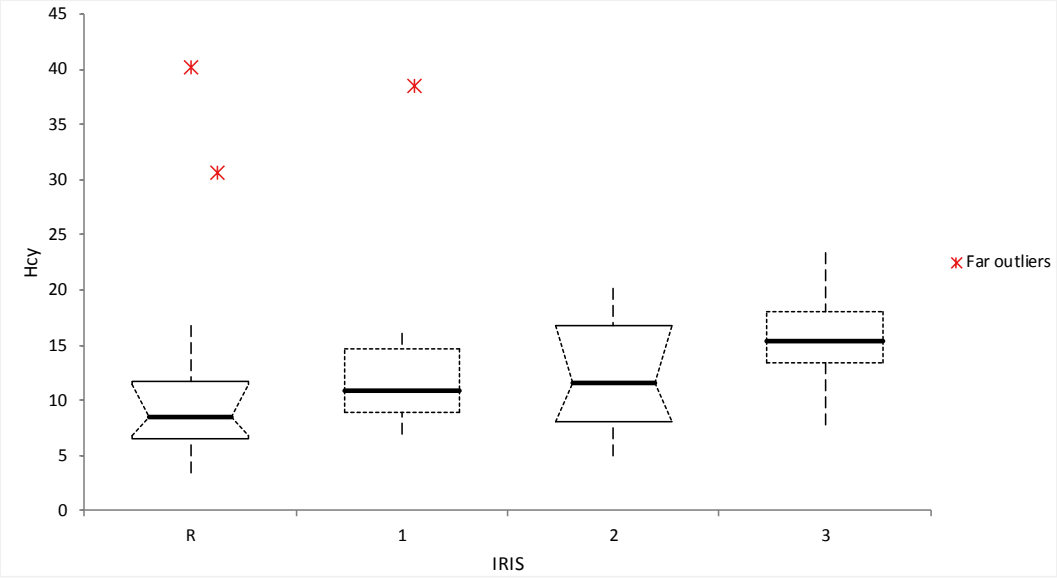


Figure 26 Graphic representation of homocysteine comparison between the at risk cats group (R) and CKD cats sub-grouped according to IRIS stages 1, 2 and 3-4 (labeled 3). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

The statistical analysis was repeated by comparing only results obtained from samples of CKD cats, in order to verify whether any absence of statistical significance could be due to the high dispersion of values in the At risk group, which also included outlier. The comparison did not show a significant difference (P = 0.319) and results are shown in Table 32 and graphically represented in Figure 27.

Table 32 Descriptive statistics of results obtained in CKD cats sub-grouped according to IRIS stage (stage 3 and 4 joined). Mean, median and Min-Max of homocysteine values are shown.

Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
1	8	14,20 (9,70-18,71) ± 10,19	10,85 (6,96-38,50)	6,96-38,50 (8,88-14,58)
2	18	12,27 (9,27-15,27) ± 4,62	11,50 (8,04-16,70)	4,94-20,10 (8,02-16,73)
3-4	10	15,48 (11,45-19,51) ± 4,77	15,40 (9,10-21,10)	7,72-23,40 (13,41-17,98)

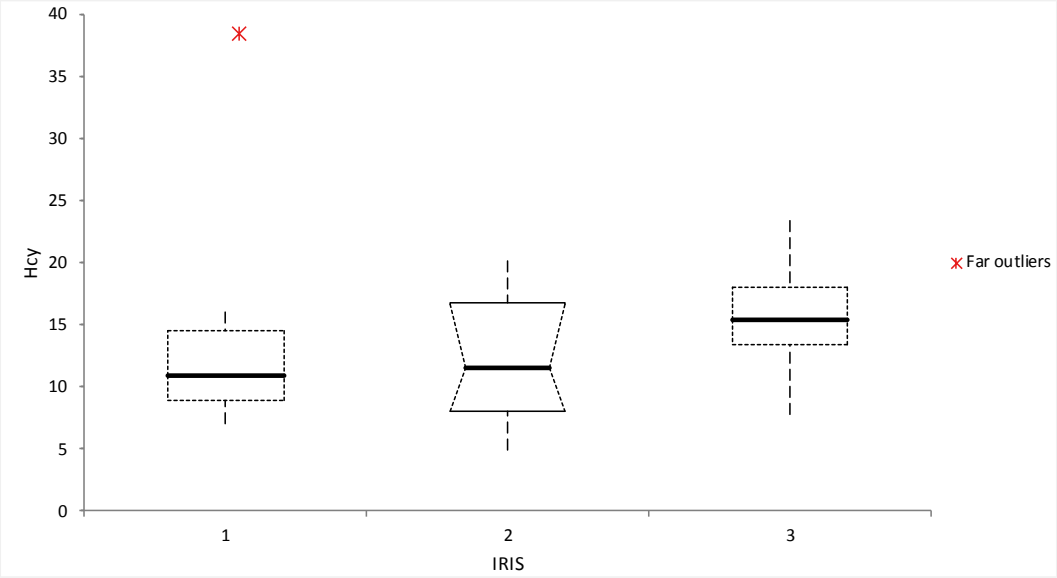


Figure 27 Graphical representation of the comparison between IRIS 1, IRIS 2 and a single group IRIS 3 and IRIS 4 (IRIS 3). Axis axes = IRIS staging; axis of the ordinates = homocysteine (Hcy) values. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

In order to verify the hypothesis that the lack of statistical differences between groups could be due to the treatments received after the first visit, all the above analysis was repeated using only the results obtained at time 0.

However, this analysis did not show significant differences (P = 0.163). Results are shown in Table 33 and graphically represented in Figure 28.

Table 33 Descriptive statistics of results of cats at risk (A) and CKD cats (N) including only samples collected at time zero. Mean, median and Min-Max of homocysteine values are shown.

Group	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
A	17	12,15 (8,59-15,71) ± 9,57	9,15 (6,82-13,30)	3,37-40,20 (6,35-13,77)
N	21	12,46 (9,26-15,66) ± 4,58	11,30 (8,62-15,10)	6,07-23,40 (8,43-15,33)

SD= standard deviation; CI= confidence interval. A= At risk; N= nephropatic cats

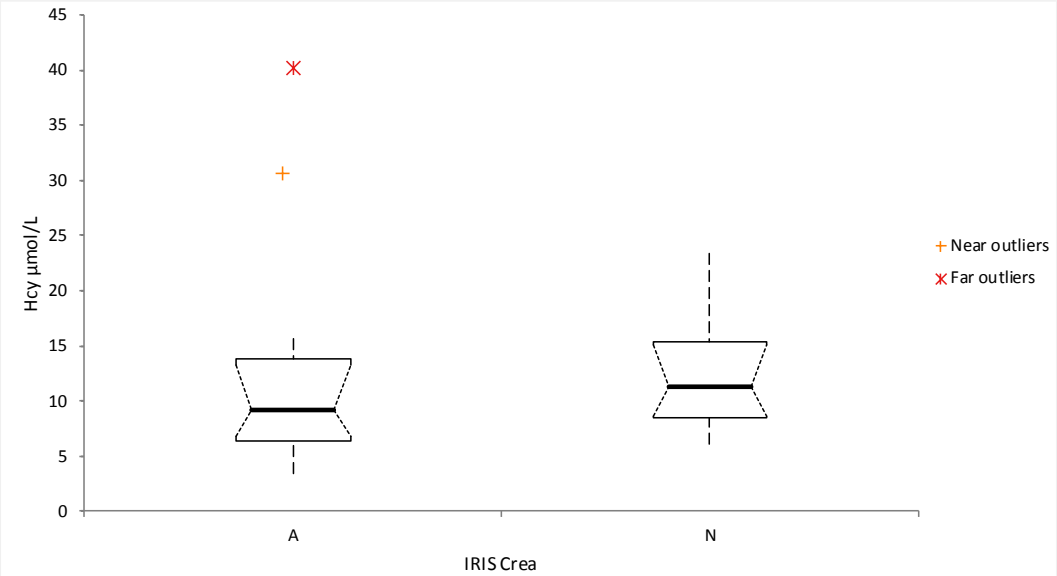


Figure 28 Graphical representation of the comparison between cats at risk and CKD cats. Axis axes = IRIS staging; axis of the ordinates = homocysteine (Hcy) values. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

Similarly, the comparison between groups divided according to IRIS stages, including samples collected at time 0 (descriptive in Table 34 and graphical representation in Figure 29), did not reveal significant differences between the groups (P = 0.168).

Table 34 Descriptive statistics of results of cats grouped according to IRIS stage, including only samples collected at time zero. Mean, median and Min-Max of homocysteine values are shown.

Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
A	17	12,15 (8,61-15,69) ± 9,57	9,15 (6,82-13,30)	3,37-40,20 (6,35-13,77)
1	5	11,78 (5,26-18,31) ± 2,75	10,90 (-)	8,62-16,00 (10,07-13,73)
2	10	10,49 (5,87-15,11) ± 3,46	10,29 (7,20-15,10)	6,07-16,70 (7,77-12,35)
3-4	6	16,30 (10,34-22,26) ± 5,50	15,40 (7,72-23,40)	7,72-23,40 (14,21-21,29)

A= at risk; SD= standard deviation; CI= confidence interval

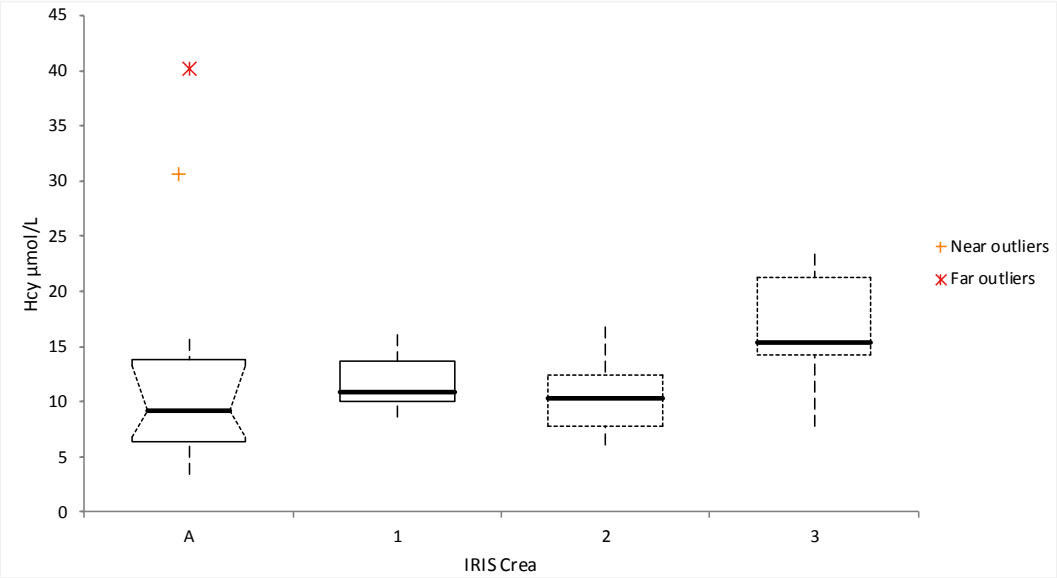


Figure 29 Graphic representation of homocysteine comparison between the At risk group (A) and CKD cats sub-grouped according to IRIS stages 1, 2 and 3-4 (labeled 3). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

The correlation between creatininemia and homocysteinemia (Figure 30) was statistically significant (P <0.001), although the correlation coefficient was moderate (r = 0.433).

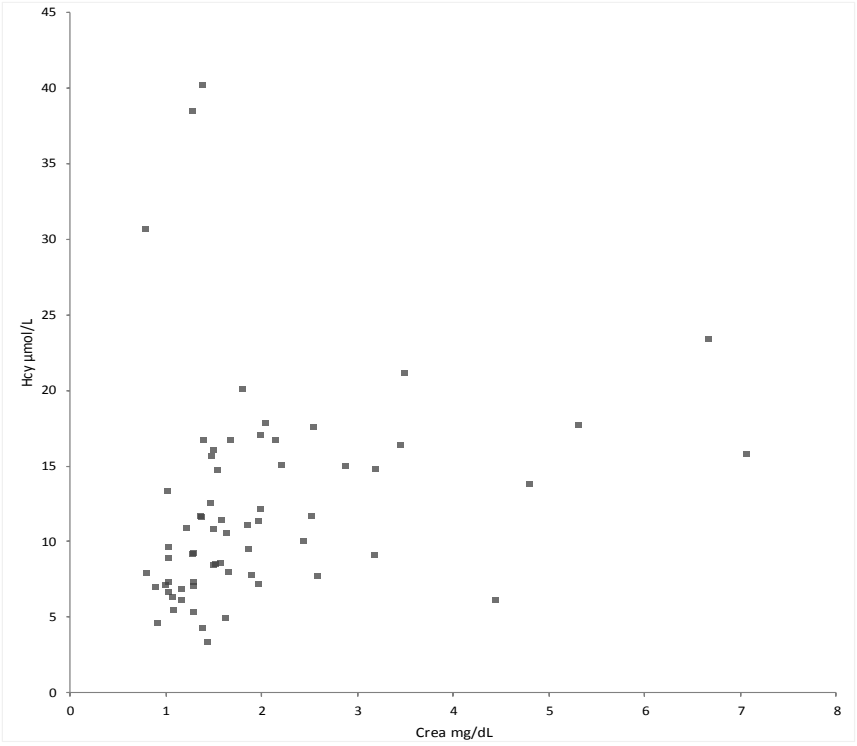


Figure 30 Graphic representation of the correlation between serum creatinine (Crea) and homocysteine (Hcy).

This result suggested that the decrease of GFR may play a role in determining the increase of concentration of homocysteinemia, despite other factors involved in the onset of hyperomocysteinemia could not be excluded.

Comparison based on proteinuria

Descriptive statistics of Hcy in groups divided according to IRIS stage for proteinuria in Table 35 and summarized in Figure 31. No statistically significant difference (P = 0.090) was found in this comparison.

Table 35 Descriptive statistics of results grouped according to IRIS sub-stage for proteinuria. Mean, median and Min-Max of homocysteine values are shown.

Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
NP	40	11,01 (8,75-13,26) ± 6,17	9,13 (7,72-12,10)	3,37-38,50 (7,14-14,76)
BP	13	15,12 (11,16-19,07) ± 10,00	11,70 (6,69-17,60)	5,42-40,20 (9,30-17,00)
P	6	15,38 (9,55-21,21) ± 5,63	13,35 (9,98-23,40)	9,98-23,40 (11,01-21,29)

NP= non-proteinuric; BP= borderline proteinuric; P= proteinuric; SD= standar deviation; CI= confidence interval

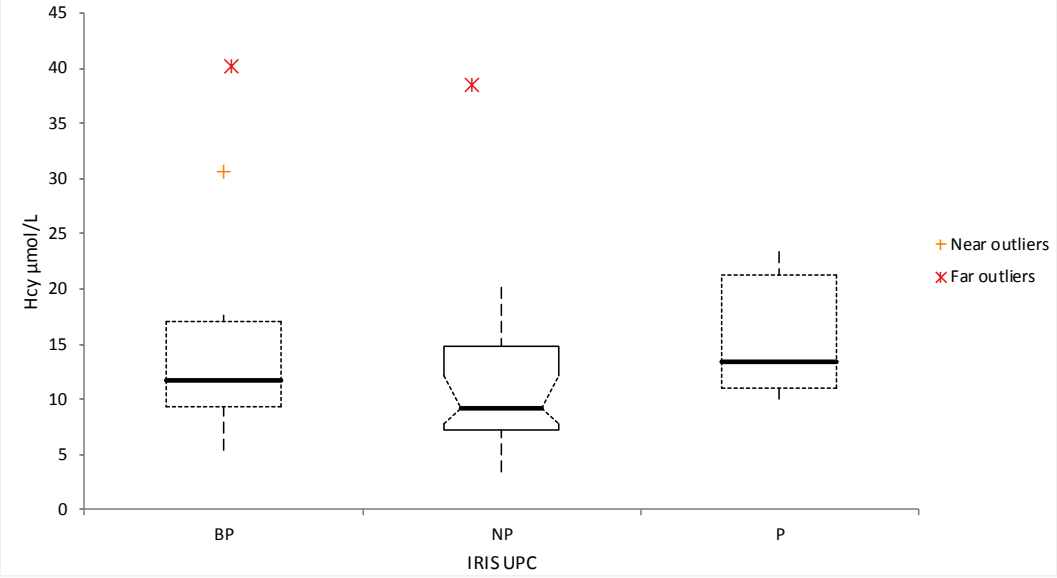


Figure 31 Graphic representation of the comparison between the group of cats divided based on the IRIS sub-stage of proteinuria (BP= borderline proteinuric, NP=non-proteinuric, P= proteinuric). Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule

Repeating the statistical analysis only on cats with CKD (Table 36, Figure 32), the difference between the groups was not statistically significant (P = 0.679).

Table 36 Descriptive statistics of results grouped according to IRIS sub-stage for proteinuria including only cats with CKD. Mean, median and Min-Max of homocysteine values are shown.

Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
NP	20	13,22 (10,18-16,26) ± 7,38	12,35 (7,82-16,00)	4,94-38,50 (7,76-16,23)
BP	5	14,36 (8,28-20,44) ± 3,27	15,80 (-)	10,80-17,60 (10,87-17,00)
P	6	15,38 (9,83-20,93) ± 5,63	13,35 (9,98-23,40)	9,98-23,40 (11,01-21,29)

NP= non-proteinuric; BP= borderline proteinuric; P= proteinuric; SD= standar deviation; CI= confidence interval

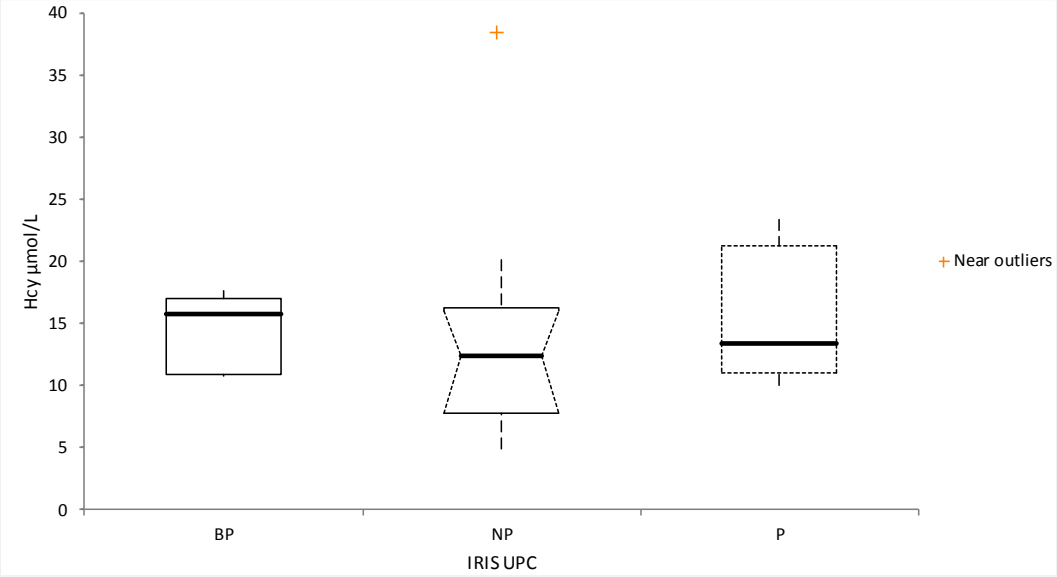


Figure 32 Graphic representation of the comparison between the group of cats with CKD divided based on the IRIS sub-stage of proteinuria (BP= borderline proteinuric, NP=non-proteinuric, P= proteinuric). Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

The difference becomes statistically significant (P = 0.011) if the comparison based on the UPC values is restricted to samples referred to the cats sampled at time 0. Comparison between the single groups revealed significant difference only between BP and NP (P = 0.025), with higher values obtained in BP group, but not between BP and P and NP and P. Results are reported in Table 37 and graphically represented in Figure 33.

Table 37 Descriptive statistics of results grouped according to IRIS sub-stage for proteinuria including only sample collected at the time of inclusion. Mean, median and Min-Max of homocysteine values are shown.

Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
NP	22	9,76 (6,93-12,60) ± 3,77	9,01 (7,20-12,60)	3,37-16,00 (7,17-12,78)
BP	10	16,61 (12,40-20,82) ± 10,64	12,50 (10,60-30,70)	5,42-40,20 (10,78-17,87)
P	4	17,65 (11,00-24,30) ± 5,62	18,05 (-)	11,10-23,40 (12,73-22,44)

NP= non-proteinuric; BP= borderline proteinuric; P= proteinuric; SD= standar deviation; CI= confidence interval

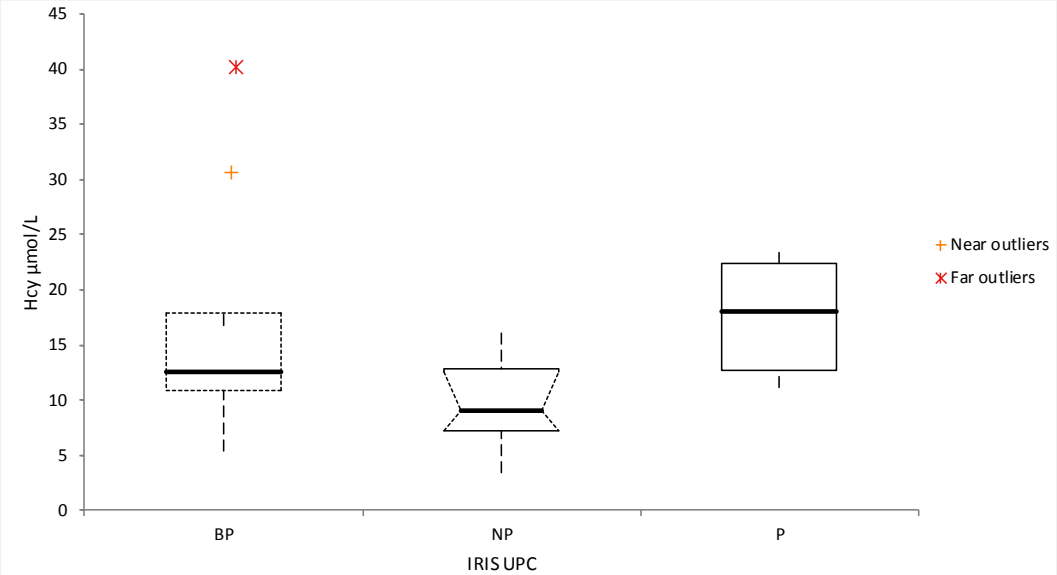


Figure 33 Graphic representation of the comparison between the group of cats divided based on the IRIS sub-stage of proteinuria (BP= borderline proteinuric, NP=non-proteinuric, P= proteinuric) including only samples collected at the time of inclusion. Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

The correlation between UPC and homocysteinemia (Figure 34) was not statistically significant (P <0.058) and the correlation coefficient was very low (r = 0.248).

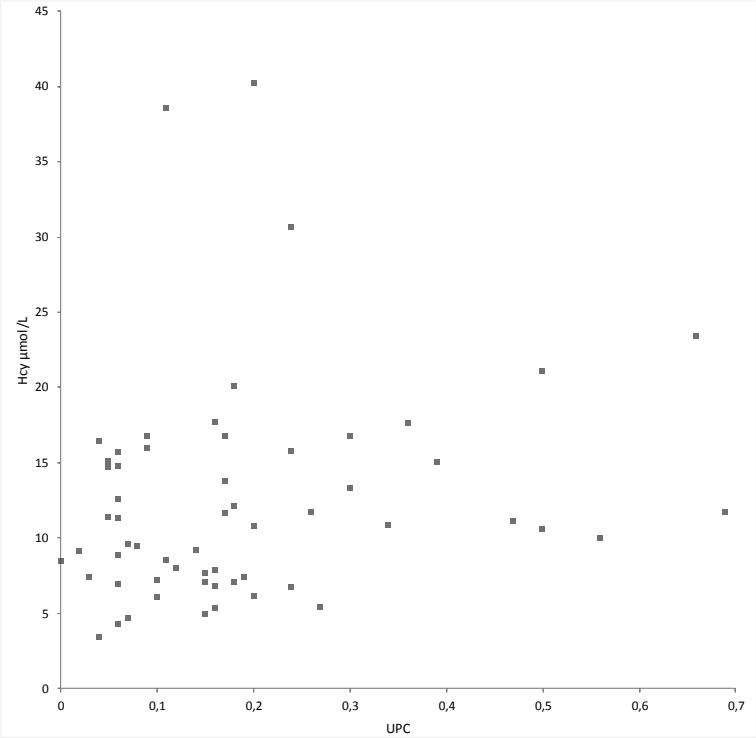


Figure 34 Graphical representation of the correlation between urinary protein-to-creatini (UPC) ratio and homocysteine (Hcy).

Comparison based on SBP

Hcy was not significantly different between groups of samples classified according to SBP (P = 0.988). Descriptive statistics as shown in Table 38 and graphically displayed in Figure 35.

Table 38 Descriptive statistics of results grouped according to IRIS sub-stage for SBP. Mean, median and Min-Max of homocysteine values are shown.

IRIS stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
AP0	32	11,42 (9,02-13,81) ± 6,93	10,75 (7,10-12,60)	3,37-40,20 (6,84-14,33)
AP1	4	9,16 (2,39-15,93) ± 2,09	8,60 (-)	7,36-12,10 (7,64-10,87)
AP2	9	12,17 (7,66-16,68) ± 7,95	7,95 (6,96-17,70)	6,82-30,70 (7,47-15,90)
AP3	7	11,14 (6,02-16,26) ± 5,32	9,98 (4,27-20,10)	4,27-20,10 (7,71-15,13)

SD= standard deviation; CI= confidence interval; AP0: SBP< 150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP= 160-179 mmHg; AP3: SBP> 180 mmHg

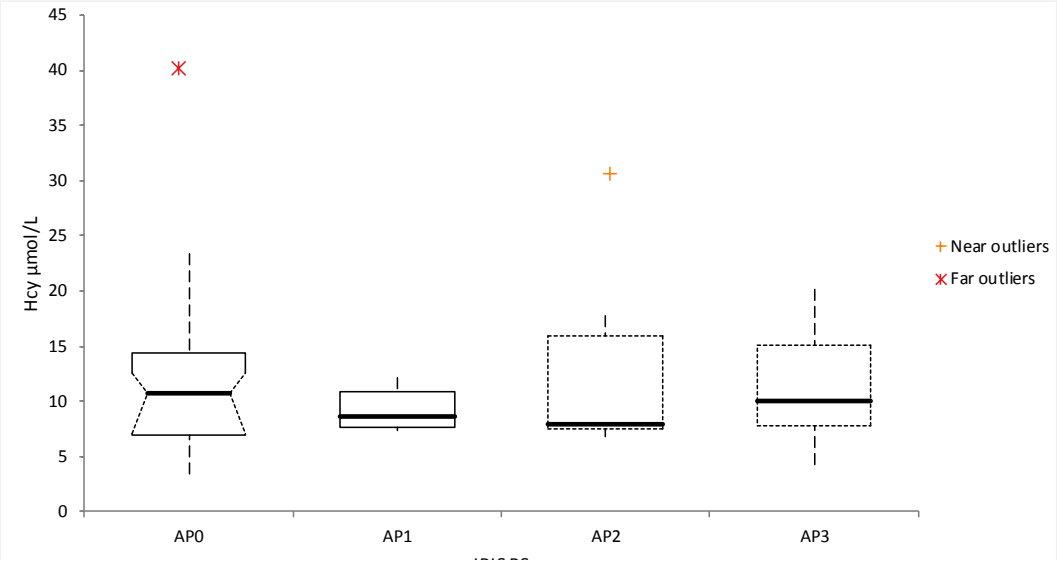


Figure 35 Graphic representation of the comparison between the group of cats divided according to the IRIS sub-stage of SBP (AP0: SBP< 150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP= 160-179 mmHg; AP3: SBP> 180 mmHg). Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

Significant differences (P = 0.985) were not observed either by comparing normotensive and hypertensive cats, using a cut-off of 150 mmHg, as shown in Table 39 and Figure 36.

Table 39 Descriptive statistics of homocysteine in hypertensive cats (HT) and normotensive cats (NT), using 150 mmHg as cut-off. Mean, median and Min-Max of homocysteine values are shown.

IRIS Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
HT	20	11,21 (8,23-14,19) ± 6,13	9,01 (7,72-12,10)	4,27-30,70 (7,51-13,79)
NT	32	11,42 (9,06-13,77) ± 6,93	10,75 (7,10-12,60)	3,37-40,20 (6,84-14,33)

SD= standard deviation; CI= confidence interval.

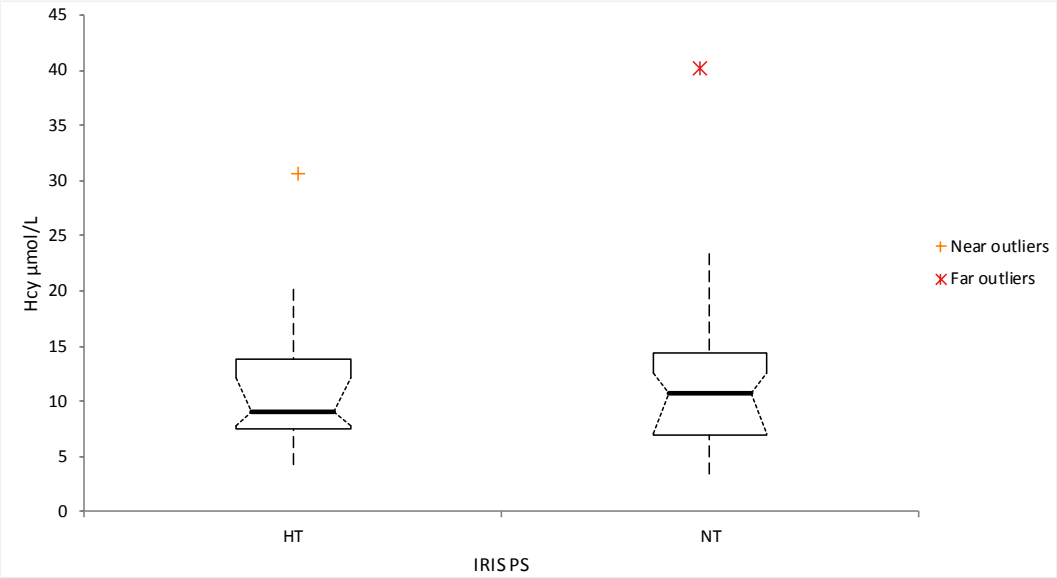


Figure 36 Graphic representation of the comparison between the hypertensive cats (HT) and normotensive cats (NT), using 150 mmHg as cut-off. Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

The same statistical analysis were performed only on the group of CKD cats in order to exclude the effect on results of the healthy cats, that in turn supposedly do not have alteration of Hcy metabolism.

However, this analysis showed neither significant differences nor comparing the different IRIS stages (P = 0.817) (Table 40 and Figure 37) neither classifying the samples as normotensive or hypertensive (using the 150 mmHg value as cut-off, P = 0.780) (Table 41 and Figure 38).

Table 40 Descriptive statistics of results grouped according to IRIS sub-stage for SBP, including only samples of cats affected by CKD. Mean, median and Min-Max of homocysteine values are shown.

IRIS Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max I-III quartile)
AP0	16	12,21 (9,79-14,63) ± 4,74	11,50 (8,62-15,80)	4,94-23,40 (8,82-15,38)
AP1	2	10,07 (3,22-16,92) ± 2,87	10,07 (-)	8,04-12,10 (8,04-12,10)
AP2	5	11,04 (6,71-15,37) ± 4,95	7,82 (-)	6,96-17,70 (7,47-15,90)
AP3	5	13,27 (8,94-17,60) ± 4,62	10,80 (-)	9,47-20,10 (9,81-17,37)

SD= standard deviation; CI= confidence interval; AP0: SBP< 150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP= 160-179 mmHg; AP3: SBP> 180 mmHg

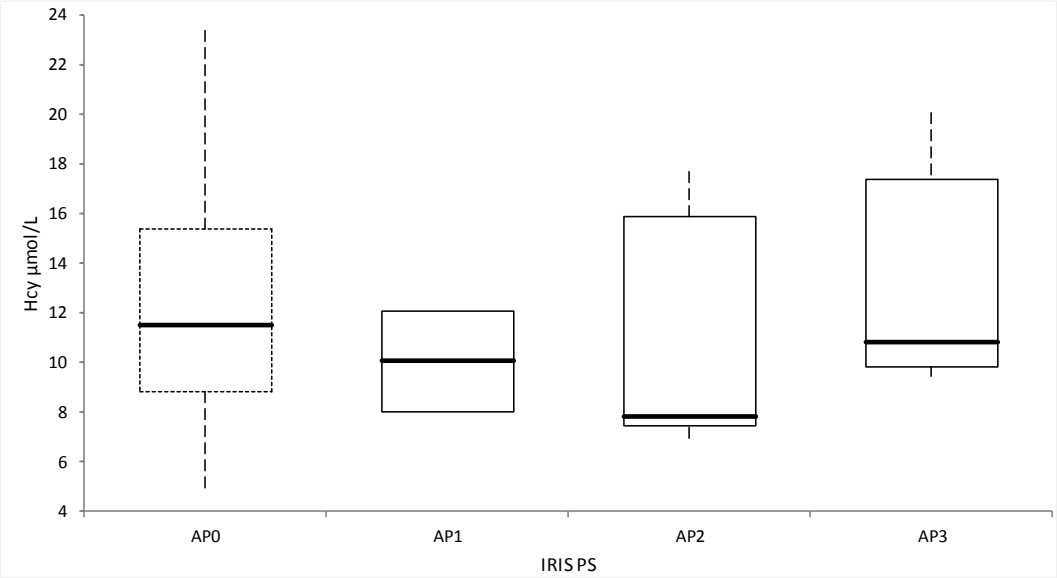


Figure 37 Graphic representation of the comparison between the group of cats divided according to the IRIS sub-stage of SBP (AP0: SBP< 150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP= 160-179 mmHg; AP3: SBP> 180 mmHg), including only samples of cats affected by CKD. Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

Table 41 Descriptive statistics of homocysteine in hypertensive cats (HT) and normotensive cats (NT), including only samples of cats affected by CKD. Mean, median and Min-Max of homocysteine values are shown.

IRIS Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
HT	12	11,81 (9,08-14,53) ± 4,38	10,39 (7,82-16,00)	6,96-20,10 (7,91-15,58)
NT	16	12,21 (9,85-14,57) ± 4,74	11,50 (8,62-15,80)	4,94-23,40 (8,82-15,38)

SD= standard deviation; CI= confidence interval.

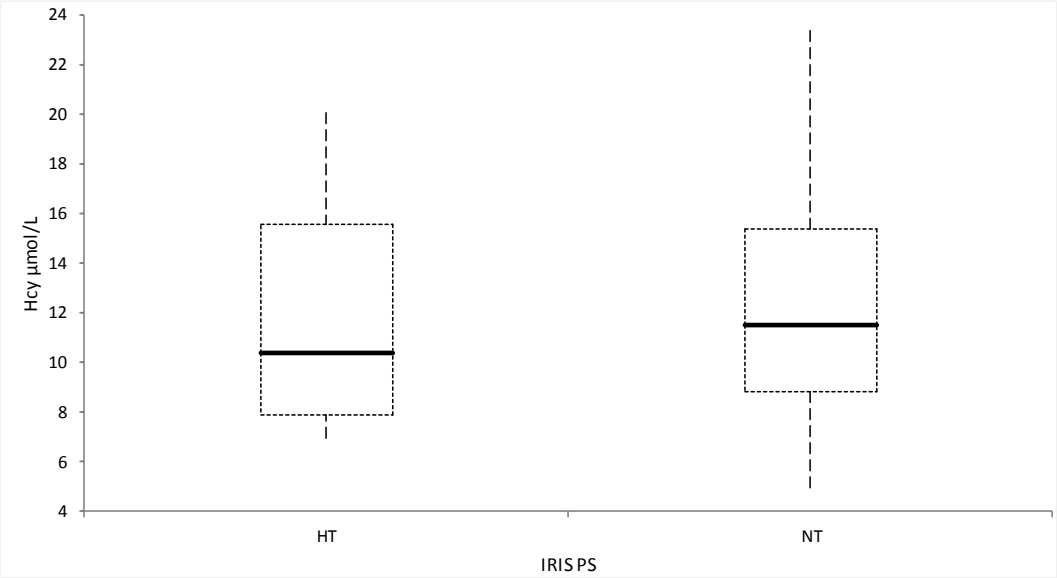


Figure 38 Graphic representation of the comparison between the hypertensive cats (HT) and normotensive cats (NT), using 150 mmHg as cut-off and including only cats affected by CKD. Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

Similarly to the data of proteinuria, even for the systolic blood pressure we performed a statistical comparison on the samples obtained at time 0 prior to each therapy. In this case, no significant differences were observed neither by comparing the different IRIS classes (P = 0.814) (Table 42 and Figure 39) nor classifying the samples as normotensive or hypertensive (using the 150 mmHg value as cut-off, P = 0.779) (Table 43 and Figure 40).

Table 42 Descriptive statistics of results grouped according to IRIS sub-stage for SBP, including only samples collected at time of inclusion. Mean, median and Min-Max of homocysteine values are shown.

IRIS Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
AP0	18	12,33 (8,51-16,15) ± 8,42	11,00 (7,20-14,70)	3,37-40,20 (7,11-14,71)
AP1	3	9,76 (0,40-19,12) ± 2,10	9,15 (-)	8,04-12,10 (8,23-11,61)
AP2	7	12,12 (6,00-18,25) ± 8,64	7,95 (6,82-30,70)	6,82-30,70 (7,74-13,98)
AP3	3	12,09 (2,73-21,45) ± 3,45	10,80 (-)	9,47-16,00 (9,69-15,13)

SD= standard deviation; CI= confidence interval; AP0: SBP< 150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP= 160-179 mmHg; AP3: SBP> 180 mmHg

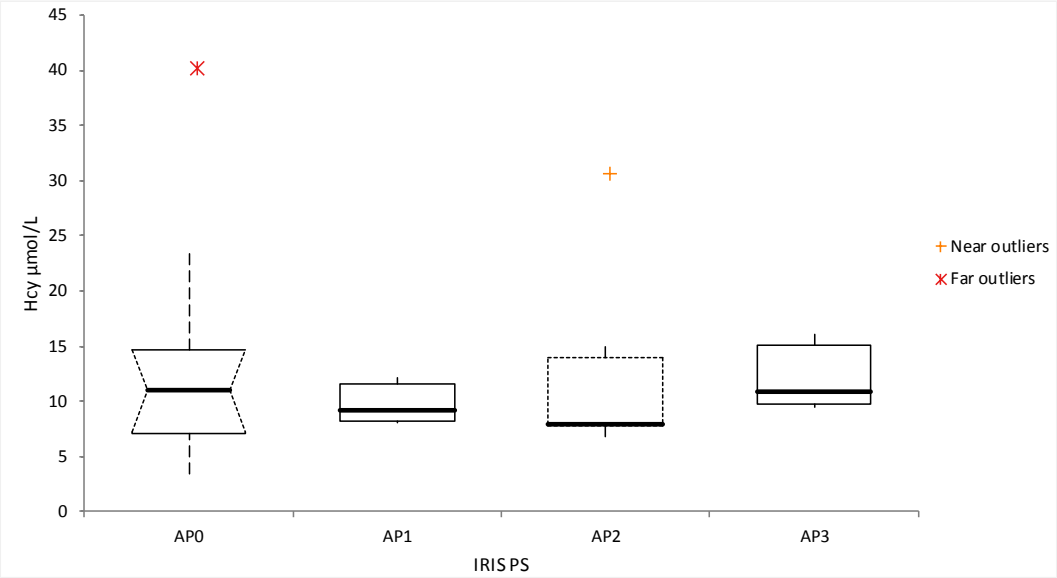


Figure 39 Graphic representation of the comparison between the group of cats divided according to the IRIS sub-stage of SBP (AP0: SBP< 150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP= 160-179 mmHg; AP3: SBP> 180 mmHg), including only samples collected at time of enrolment. Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

Table 43 Descriptive statistics of homocysteine in hypertensive cats (HT) and normotensive cats (NT), including only samples collected at time of inclusion. Mean, median and Min-Max of homocysteine values are shown.

IRIS Stage	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
HT	13	11,57 (7,23-15,91) ± 6,41	9,15 (7,82-15,00)	6,82-30,70 (7,91-13,07)
NT	18	12,33 (8,65-16,02) ± 8,42	11,00 (7,20-14,70)	3,37-40,20 (7,11-14,71)

SD= standard deviation; CI= confidence interval.

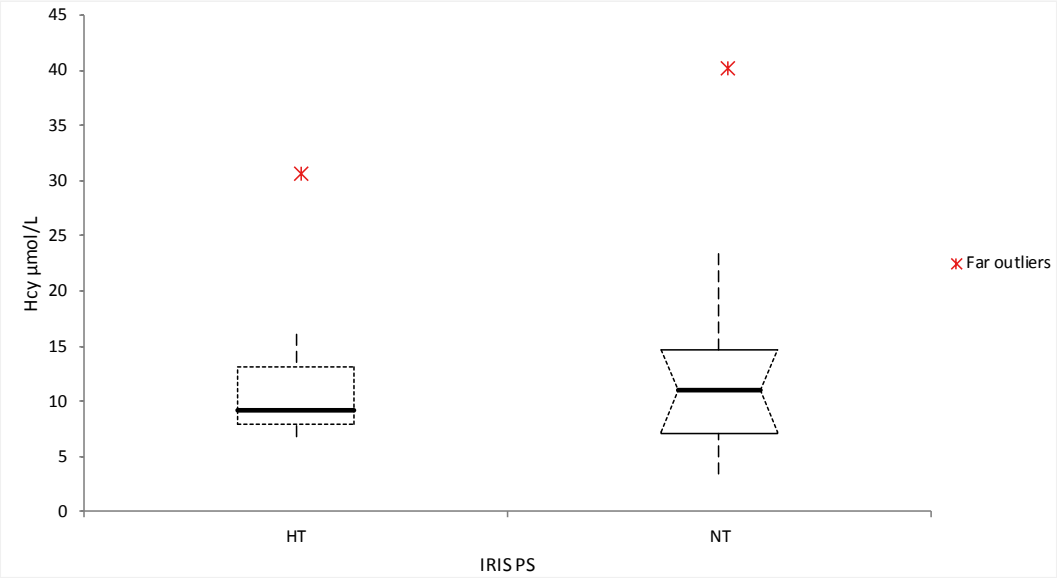


Figure 40 Graphic representation of the comparison between the hypertensive cats (HT) and normotensive cats (NT), using 150 mmHg as cut-off and including only samples collected at time of enrolment. Axis axis = IRIS staging; order axis = amount of homocysteine (Hcy). Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

Furthermore, no significant correlation (P = 0.964; r = -0.006) was found between BP and homocysteinemia levels (Figure 41).

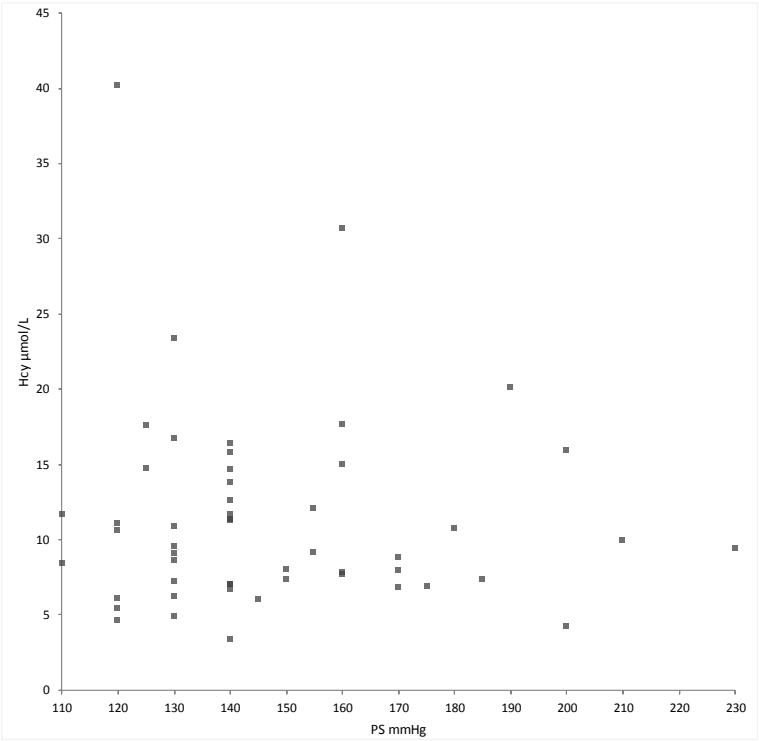


Figure 41 Graphical representation of the correlation between systolic blood pressure (PS) and homocysteine (Hcy).

Disease progression

Regardless of the mechanism responsible for hyperomocysteinemia in cats with CKD, we checked if the Hcy could be an early marker of renal disease despite the inappropriate experimental design and the few data available on the follow up of the cats included in the study. Therefore, the cats at risk were divided into groups depending on whether or not they had developed CKD within 6 to 12 months after the first sample collection.

This comparison, shown in Table 44 and graphically represented in Figure 42, did not reveal a significant difference between the groups (P = 0.384), although mean and median values of Hcy were lower in cats that did not develop CKD compared to those found in cats that developed kidney disease.

Table 44 Descriptive statistics of homocysteine in samples collected at time of enrolment in cats grouped depending on whether or not ("progressive" or "stable", respectively)they had developed CKD within 6 to 12 months after the first sample collection. Mean, median and Min-Max of homocysteine values are shown.including only samples collected at time of inclusion.

Group	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
Progressive	6	14,84 (10,13-19,56) ± 8,38	13,65 (7,20-30,70)	7,20-30,70 (8,72-16,31)
Stable	12	10,85 (7,51-14,18) ± 3,36	11,05 (7,95-13,30)	5,42-16,70 (8,45-12,80)

SD= standard deviation; CI= confidence interval.

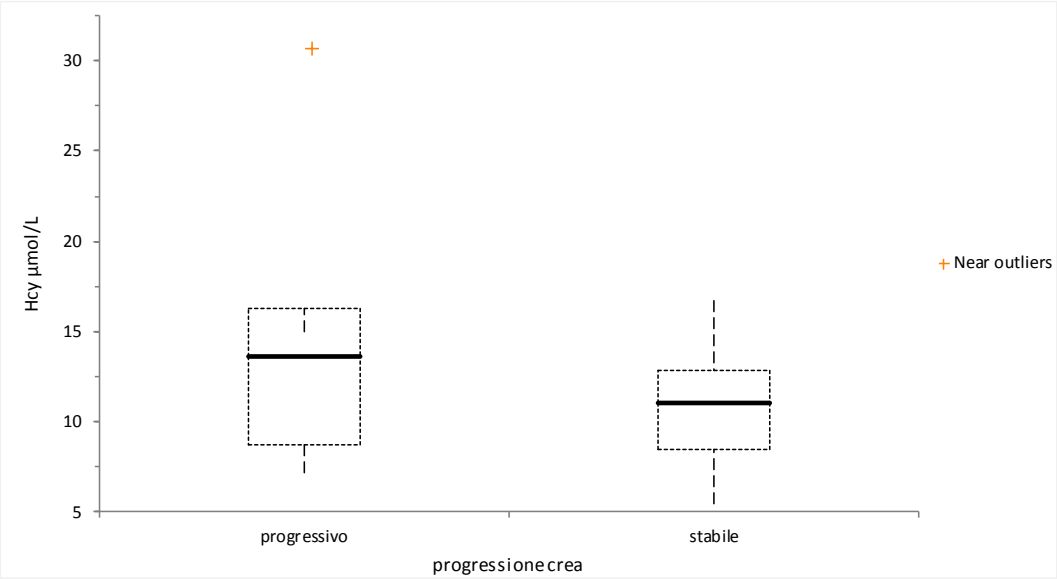


Figure 42 Graphic representation of the distribution of homocysteine in samples collected at time of enrolment in cats grouped depending on whether or not ("progressive" or "stable", respectively) they had developed CKD within 6 to 12 months after the first sample collection. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

The previous analysis was repeated including only At risk cats. The results, shown in Table 45 and represented in Figure 43, showed that although there is no statistical significance (P = 0.110).

Table 45 Descriptive statistics of homocysteine in samples collected at time of enrolment in cats at risk grouped depending on whether or not ("progressive" or "stable", respectively) they had developed CKD within 6 to 12 months after the first sample collection. Mean, median and Min-Max of homocysteine values are shown.

Group	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
Progressive	3	18,09 (9,22-26,95) ± 11,31	14,70 (-)	8,86-30,70 (9,83-28,03)
Stable	6	9,69 (3,42-15,96) ± 2,81	9,88 (5,42-13,3)	5,42-13,30 (7,74-11,83)

SD= standard deviation; CI= confidence interval.

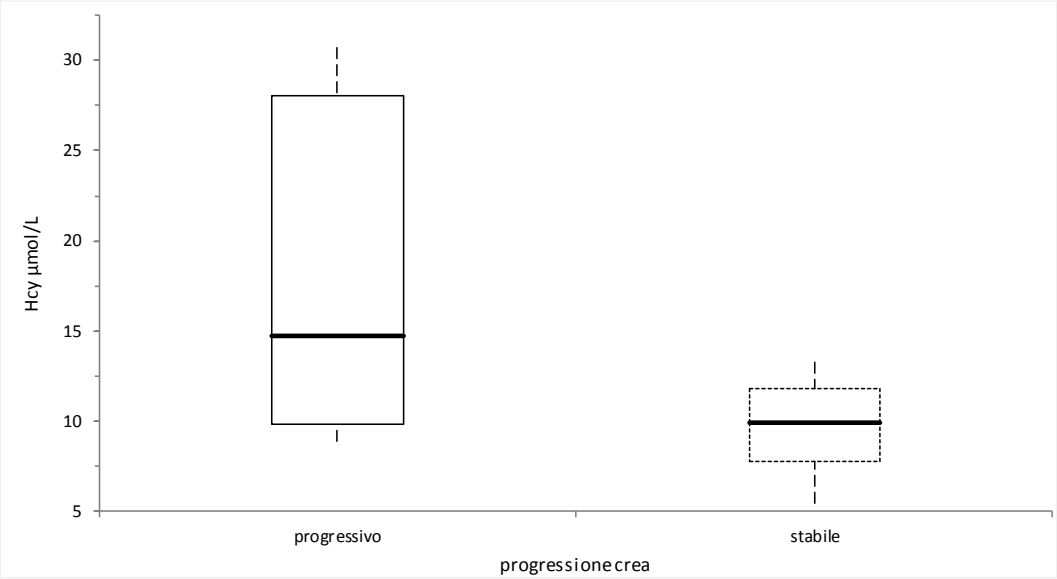


Figure 43 Graphic representation of the distribution of homocysteine in samples collected at time of enrolment in cats at risk grouped depending on whether or not ("progressive" or "stable", respectively) they had developed CKD within 6 to 12 months after the first sample collection. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

To verify whether the Hcy values at T0 were predictive of the development of proteinuria and hypertension, the comparison between stable and progressive patients was performed using this two IRIS sub-staging, irrespective of the degree of CKD (i.e. IRIS stage based on serum creatinine),

Regarding proteinuria, there were no significant differences between stable and progressive patients (P = 0.304) and no tendencies to increase mean or median Hcy values Table 46 and in Figure 44). As previously done for creatinine, it was not possible to repeat this comparison only on subjects at risk, because none of them showed a transition to a higher stage of proteinuria than that found at T0.

Table 46 Descriptive statistics of homocysteine in samples collected at time of enrolment in cats grouped depending on whether or not ("progressive" or "stable", respectively) they had developed proteinuria within 6 to 12 months after the first sample collection. Mean, median and Min-Max of homocysteine values are shown.

Group	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
Progressive	2	8,69 (0,59-17,96) ± 3,70	8,69 (-)	6,07-11,30 (6,07-11,30)
Stable	13	12,71 (9,07-16,35) ± 6,23	12,10 (7,95-15,00)	5,42-30,70 (8,56-14,80)

SD= standard deviation; CI= confidence interval.

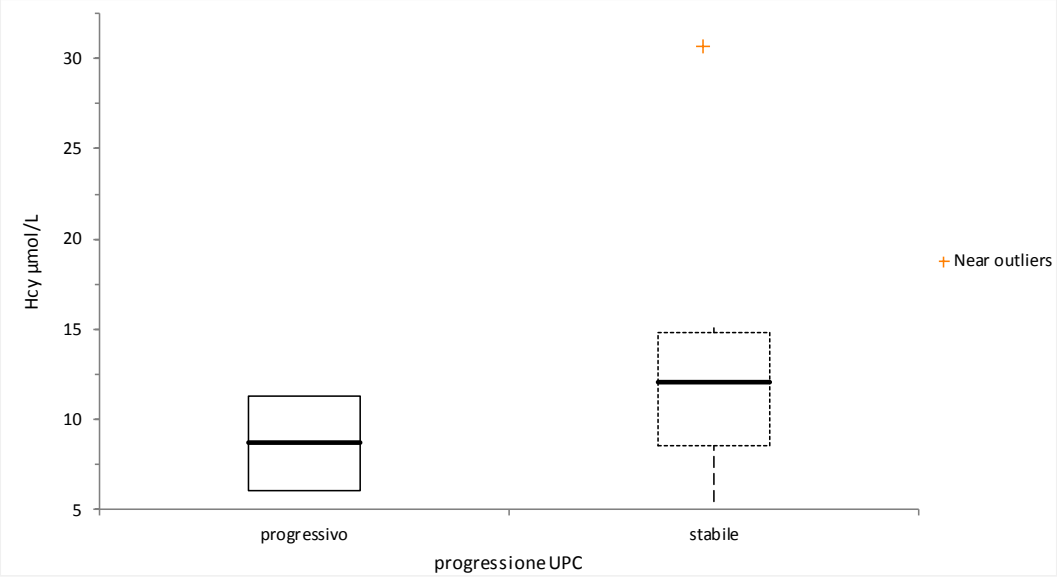


Figure 44 Graphic representation of the distribution of homocysteine in samples collected at time of enrolment in cats grouped depending on whether or not ("progressive" or "stable", respectively) they had developed proteinuria within 6 to 12 months after the first sample collection. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

Similarly, no significant difference (P = 0.481) in Hcy values at time 0 was found between cats who maintain normal SBP and cats which then showed an increase in the IRIS staging of hypertension at subsequent sample collections (Table 47, figure 45). However, similar to that found for creatinine-based IRIS, a tendency to increase median and median values in progressive patients has been observed. Therefore, it could be interesting to perform future studies with multiple sampling and higher sample of patients, in order to check whether this preliminary visual difference can play a prognostic role. This type of observation could be relevant both in CKD and in at risk subjects, but in this study a separate comparison within the two groups was not possible because only a cat with CKD and only two of the risk cats showed increasing SBP values.

Table 47 Descriptive statistics of homocysteine in samples collected at time of enrolment in cats grouped depending on whether or not ("progressive" or "stable", respectively) they had developed hypertension within 6 to 12 months after the first sample collection. Mean, median and Min-Max of homocysteine values are shown.

Group	N	Mean (95% CI) ± SD	Median (95% CI)	Min-Max (I-III quartile)
Progressive	3	11,62 (7,57-15,67) ± 2,93	11,30 (-)	8,86-14,70 (9,27-14,13)
Stable	9	9,52 (7,18-11,86) ± 3,20	9,15 (6,07-12,60)	5,42-15,00 (6,82-12,00)

SD= standard deviation; CI= confidence interval.

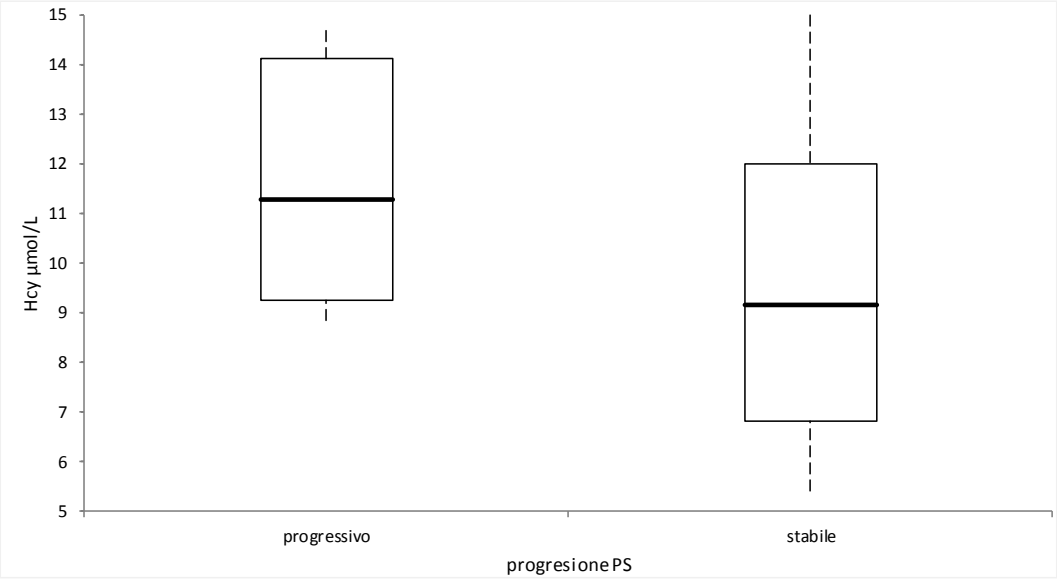


Figure 45 Graphic representation of the distribution of homocysteine in samples collected at time of enrolment in cats grouped depending on whether or not ("progressive" or "stable", respectively) they had developed hypertension within 6 to 12 months after the first sample collection. Boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median value, the vertical lines are the limits of suspected outlier distribution according to the Tukey rule.

Discussion

The validation tests showed that the determination of Hcy in the cat with the enzymatic method had more than acceptable levels of imprecision and accuracy, analogously to study performed on other species (Rossi et al., 2008).

Overall, intra-assay results showed a satisfactory performance, highlighting lower CVs with higher Hcy values and higher CV at lower Hcy values. This result is not surprising, since it is already known that when the concentration of an analyte in a sample is low, the slight shift of a measurement from the mean value determines a more profound change of CV compared to a sample with higher concentration (Westgard, 2003).

Also the inter-assay CV was acceptable, even if it was higher than that found in the intra-assay tests. Higher variability is expected with inter-assay test since, in addition to the intrinsic variability of method, other factors (e.g. the preservation of the sample) can affect results (Kale et al., 2012).

The results of the LUD test showed that the method, applied on feline serum, had excellent accuracy, similar to that observed in other species (Rossi et al., 2008).

The most diluted sample has always showed values below the detection limit of the method, suggesting that even at low values the test seems to be very repeatable and on the other hand that the dilution accuracy is high.

A more complete assessment of accuracy would require a comparison of results with those obtained using a gold standard, which is not available for the feline species, and the performance of the spiking tests. This last test should be carried out by measuring the concentration of Hcy in the serum before and after the addition of purified feline Hcy (not available on the market) or more commonly, by quantifying the analyte in a serum with low concentration, supplemented with increasing volume of another serum in which the concentration of the analyte is higher. In our study, this test could have been done by adding high Hcy concentration pools (used in imprecision and LUD tests) to the low-concentration Hcy pool used in the imprecision test. However, in cats this kind of evaluations is conditioned by the low volumes of serum that can be collected from each animal and in the majority of cases available volume is not sufficient to perform all the tests required in routine method validation (Kjelgaard-Hansen and Jensen, 2010). Given that, our good dilutions test results and the necessity to prepare other serum pool for the spiking recovery tests with the risk of decreasing the number of samples useful for subsequent analyzes, we decided to not "consume" other serum aliquots to perform this additional test. For the same reasons, other tests commonly included in validation studies, such as the evaluation of quantification and detection limits (Kjelgaard-Hansen and Jensen, 2010), conservation analysis at different time and temperature conditions, or evaluation of the interference of substances such as lipids, hemoglobin and jaundice, were not performed.

However in dogs, although some interferers had statistically significant variations on the expected values, no clinically relevant variations were observed neither in the presence of interferents at concentrations commonly found in clinical practice or after conservation for medium to long time (Rossi et al., 2008). Therefore, it could be axpected that this also could occur in the cat.

Given this, the method provides results that are easily interpretable also in case of small differences between the groups. When samples exhibit macroscopic alterations due to severe hemolysis, jaundice or lipemia, or samples with extreme conservation conditions are used, results should be interpreted with caution.

Overall, the results obtained from the comparison of the groups classified basing on creatinine and on the IRIS stage showed an increase in CKD cats in comparison with the cats at risk. Moreover, in the CKD group, a progressive increasing trend comparable to the IRIS stage was shown, although not always the observed differences appeared to be significant. This result could be due to a certain degree of overlapping results between the

different groups, and to the number of samples in groups, not always sufficiently high, that could have masked significant differences.

From a pathogenetic point of view, the increase of Hcy in nephropatic cats could have difference causes. Since Hcy is mainly excreted by the kidney, the reduction of GFR could reduce the excretion of this amino acid (Selhub, 1999). Also, it was hypothesized that Hcy metabolism is directly or indirectly affected by CKD (van Guldener, 2006).

The trend of Hcy to increase only at late stage of CKD represents a difference with what previously found in dogs. Indeed Rossi and colleagues showed that Hcy was higher early at stage 2 compared to control dogs and IRIS stage 1, whereas no significant differences were found between IRIS 2 and the higher values (Rossi et al., 2013b). A possible explanation of this different behavior of Hcy in cats compared to dogs could be due to its different metabolic features in this specie. Indeed, Hcy in cats is not affected by enteropaties (McMichael et al., 2000) and by cobalamin deficiency (Ruaux et al., 2001). An other possible explanation is the different characteristics of the healthy patients in the two studies: in the study in dogs young healthy patients were included (Rossi et al., 2013b) whereas in our study old cats were selected. Whether age affect Hcy concentration have to be determined in cats. Moreover, since slow progressive CKD is common in elderly cats, it is not possible to exclude that

The value obtained from cats At risk (mean ±SD: 8.74 ±3.48 µmol/L) can to be considered "normal" for healthy cats at risk to develop CKD. However, it should be considered that this result cannot be extended to the entire feline population at risk, due to the small dimension of the population considered in this study, also to the not comparable collection times and also to the application of not strictly selection criteria of well-defined statistical approaches, which are based on the observed distribution of values, as recommended by the guidelines for the definition of reference ranges elaborated by the American Society of Veterinary Clinical Pathology (Friedrichs et al., 2012). Therefore in the future, we will focus the study using the reference range in accordance with the above guidelines.

Despite these considerations, these results suggested that normal levels in cat are slightly superior to those found in normal dogs (mean ±SD: 5.72 ±3.26 µmol/L) in a previous study (Rossi et al., 2008). However, it is interesting to note that dogs included in control group had lower mean age (mean ±SD of age: 5.7 ±2.3 years) compared to healthy cats in our study (11.1 ±2.7 years, data not shown). This higher values in healthy cats could have affect statistical significance in comparison with CKD cats. Since CKD is common in

elderly cats, it is not possible to exclude that a mild slow-progressive CKD was present in some of cats at risk.

Preliminary data obtained from our study suggested that high Hcy in a cat without any signs of pathologies that can potentially induce hyperomocysteinaemia may be predictive of the occurrence of CKD pathologies. However, this study is not designed to determine the prognostic value of Hcy in feline CKD and sampling number of the groups was particularly small.

It is also important to note that some patients who developed CKD in the following months after the sample collection yielded Hcy concentration similar to stable patients.

Overall, results obtained in the analysis based on degree of proteinuria showed a modest increasing trend similar to the IRIS stages increment.

This tendency was evident in the whole case and in particular, in the samples corresponding to time of inclusion where a significant difference was found between NP and BP patients. This result could be interesting since the pathological importance of borderline proteinuria in CKD cats is still debated and the presence of hyperhocysteinemia in these patients could suggest to not underestimate borderline proteinuria in CKD cats.

Despite this significant difference that needs further evaluation, these findings, together with the low level of correlation between the degree of proteinuria and homocysteinemia, represented a further difference between dog and cat in the behavior of Hcy during CKD. However, this difference is not surprising in the light of the different pathogenesis of canine kidney disease, which is predominantly associated with immunocomplex glomerulonephritis, compared to feline CKD, which rarely glomerular injury is predominant in the pathogenesis (Polzin, 2017). Hypertension and proteinuria in the cat seem to be less associated with each other and the absence of correlation between homocysteinemia and proteinuria, together with the possible different Hcy metabolism hypnotized in other study (McMichael et al., 2000), could be a further confirmation to this assumption.

The serum Hcy concentration in the cat did not seem dependent to the SBP, unlike what happens in humans (Stanger et al., 2004). This finding was in accordance with the hypothesis of McMichael and colleagues (McMichael et al., 2000), which suggested that in the cat the serum Hcy concentration depends mainly on the peculiar metabolism in this specie. Moreover, the lack of correlation between homocysteinaemia and SBP and the absence of significant differences between groups confirmed what was already found in the dog. Specifically, a study showed no significant differences in Hcy concentration

between non-hypertensive animal or with minimal increases in SBP compared to dogs with hypertension, both considering the total number of nephropathic dogs, irrespective of the IRIS stage and within the individual IRIS stages (Rossi et al., 2013b).

4.3 Serum aldosterone

Introduction and aims

The RAAS is one of the main control system of the systemic hypertension (Mishina et al., 1998; Syme, 2011). Increased secretion of aldosterone is considered a link among renal disease, sodium retention, volume expansion, peripheral vascular resistance, and systemic hypertension (Syme, 2011). In cats, high aldosterone concentration was demonstrated with CKD and even higher values were found with concurrent CKD-related hypertension (Jepson et al., 2014). Systemic hypertension is an important potential complicating factor of CKD (Syme, 2011) and is common in cats with this disease. (Syme et al., 2002a; Kobayashi et al., 1990; Brown et al., 2007;Taylor et al., 2017). The persistence of high SBP can lead to severe clinical consequences due to target organ damage (TOD), affecting the eyes, heart, brain and the kidney itself (Sansom et al., 2004;Taylor et al., 2017). Determination of systolic blood pressure (SBP) is therefore mandatory to correctly stage and treat cats with CKD. However, diagnosis of hypertension could be challenging in cats since white coat hypertension is reported to occur frequently, with increase of SBP on average around 22 mmHg during clinical examination, but with a highly unpredictable interindividual effect and SBP increasing by as much as 75 mmHg in some cats (Belew et al., 1999).

Therefore, a biomarker of hypertension not affected by white-coat hypertension could be useful in cats, although previous studies failed to demonstrate diagnostic utility of a group of biomarkers (i.e. plasma N-Terminal probrain natriuretic peptide, vascular endothelial growth factor and cardiac troponin I) in cats to this aim (Bijsmans et al., 2017).

The aim of this study was to evaluate the value of aldosterone in cats at risk of developing CKD and in subjects with CKD. The analysis of the results will allow us to evaluate how aldosterone varies in different stages. In addition, in the case of white-coat hypertension, serum aldosterone was evaluated in hypertensive and normotensive subjects in order to evaluate its utility in distinguishing this type of hypertension from the true HT that needs treatment.

Material and methods

Selection of samples

Fifty-seven samples obtained from 35 cats were included in this study (of 22 subjects the samples at 12 months or, if not available, at 6 months were also selected).
Sample analysis was subdivided into three work sessions:
• In the first session, 10 samples (3 healthy and 7 nephropathy) with a serum volume between 1 and 1.5 mL were tested, with the aim of validate the test.
• In the second session (that was performed the following day), the intra-assay repeatability test and the linearity under dilution test were performed. These tests were considered a first validation of the ELISA kit. Moreover, the analysis of 28 additional samples was performed in this session. The intra-assay repeatability test was performed using the samples of the first session, kept refrigerated for not more than 12 hours. Specifically, based on its results and using residual serum volume, samples with lower aldosterone concentrations were mixed to form a pool (labelled "low pool") while those with higher concentrations were mixed to form a second pool (called "high pool"). Each of these two pools has been tested 5 times. The perform linearity under dilution test; a third pool with very high aldosterone concentration (using the two pool with the highest values) was prepared. This pool was serially diluted by a twofold dilution scheme (i.e 1:2, 1:4, 1:8 and 1:16) using distilled water. The expected values corresponding to each dilution were calculated. The percentage of recovery of the observed values compared with expected values at each dilution was also calculated as follow: recovery = mean observed / expected X 100.
• In the last session, 19 further samples were tested.

Measurement of Aldosterone

A commercially available ELISA kit ("Parameter Aldosterone Assay", R & D System, Minneapolis, MN, USA) was used for the determination of aldosterone. This is a competitive type of ELISA designed to measure serum, plasma, urine and cell culture supernatants. The ELISA plate was coated with anti-mouse goat IgG and the competitor aldosterone was conjugated to horseradish peroxidase. The reported minimum detectable dose was 15.2 pg/mL.
At the time of analysis, selected sera were gently thawed by transferring tubes at 4°C the day before analysis and then at room temperature one hour before analysis.

Analytical procedures were performed following the manufacturer's instruction, setting at each run standard solutions (six solutions between 6000 pg/mL and 24.7 pg/mL).
Plate was washed with an automatic washing instrument (BioRad Microtech, Segrate, Milan, Italy), aspirating and applying the wash buffer solution for a total of 4 consecutive times.
The optical density of each well was determined at 450 nm and 540 nm within 30 minutes after the addition of the stop solution. For this process a spectrophotometer (Labsystem Multiscan MS, Labsystems Helsinki, Finland) was used. A standard curve was set using commercially available software (CurveExpert Professional, 2.3.0) and results were calculated accordingly.

Statistical analysis

Statistical analysis was performed with software (GraphPad Prism 5.0, GraphPad Software, San Diego, CA, USA). For each test, a p-value of less than 0.05 indicated statistical significance. The normality of the distributions was evaluated by the Shapiro-Wilk test.
The correlation between aldosterone and serum creatinine or SBP was tested with the Spearman test, examining all the samples at all times.
Mann-Whitney's non-parametric test was used to compare aldosterone concentration among the following pairs of groups:

- Cats at risk ("At risk") vs nephropathic cats (independently of the IRIS stage, "CKD" group).
- A group of "stable" subjects vs. group of defined "progressive" subjects, based on the information obtained in the months following the inclusion; Progression was defined as passing to a higher IRIS stage.
- Group of normotensive ("NT") vs. Hypertensive ("HT") subjects; For the purposes of this study, hypertension has been defined as cats with a constant pressure of more than 150 mmHg.

The non-parametric test of Kruskal-Wallis has been used to compare aldosterone concentration in three or more groups:

- Groups divided by IRIS classification.
- Cats normotensive at risk vs. normotensive nephropathy cats ("CKD NT") and hypertensive nephropathy cats ("CKD HT").

Using the data obtained in subjects with more than one sampling over time, the nonparametric Wilcoxon signed ranks test was used to compare the concentration of aldosterone obtained at inclusion ("T0") and after 6 or 12 months ("T6-12") for both at risk and for nephropathic (CKD) subjects.

For each group median, range, I, and IV interquartile, mean, and standard deviation were calculated.

Results and discussion

Intra-assay imprecision

The results of the 5 repeated readings on the two serum pools are shown in Table 48.

Table 48 Values obtained from the intra-assay test on the "low" pool and on the "high" pool

N° of Reading	"Low Pool" (pg/mL)	"High Pool" (pg/mL)
1	133.2	223.7
2	145.9	305.3
3	129.9	314.5
4	138.4	301.7
5	144.4	247.6

The "low pool" provided an mean value of 138.2 ± 6.7 pg/mL, corresponding to a 4.8% CV. The "high pool" provided an average value of 278.6 ± 40.3 pg / mL, for a CV of 14.5%.
Linearity under dilution test demonstrated poor recovery results, yielding mean percentages of recovery compared with expected values equal to 9.6% (Table 49) and failing to linear distribution (figure 46)

Table 49 Serial dilutions of aldosterone pool with relative observed value (mean of three measurements), expected and recovery rate (O/E recovery)

Dilution	Observed (pg/mL)	Expected (pg/mL)	O/E (%)
Undiluted	4476,6	4476,6	
1:2 alto1	328,06	2238,3	14,66
1:4 alto	100,1	1119,15	8,94
1:8 alto	38,55	559,575	6,89
1:16 alto	22,25	279,7875	7,95

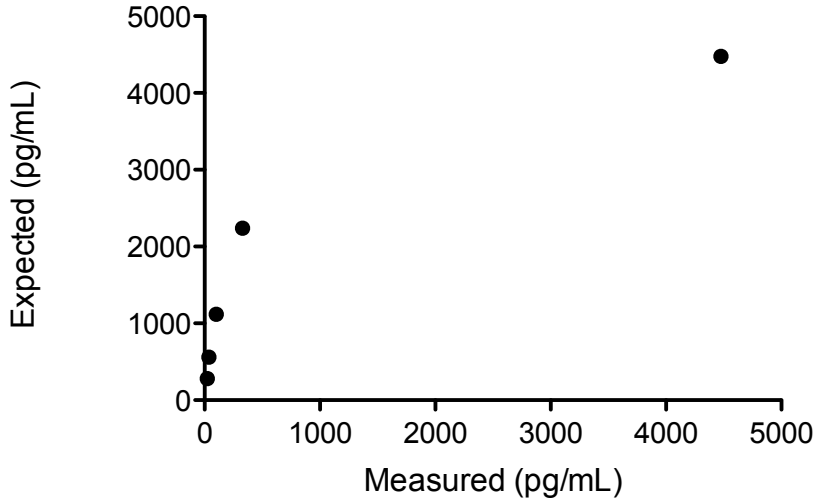


Figure 46 Graphic representation obtained from linearity under dilution tests.

Excluding the first dilution and considering the 1:2 dilution as the starting point, better recovery (mean = 84.5%) (Table 50) and an almost linear distribution were found (Figure 47).

Table 50 Serial dilutions of aldosterone pool with relative observed value (mean of three measurements), expected and recovery rate (O/E recovery), excluding the first dilution and considering the 1:2 dilution as the starting point

Dilution	Observed (pg/mL)	Expected (pg/mL)	O/E (%)
Undiluted			
1:2	328,06	328,06	
1:4	100,1	164,03	61,03
1:8	38,55	50,05	77,02
1:16	22,25	19,28	115,43

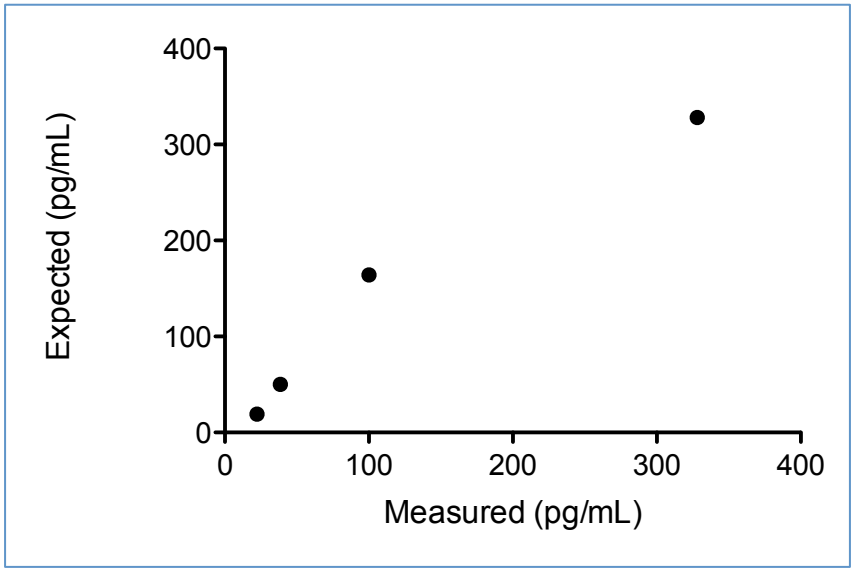


Figure 47 Graphic representation obtained from linearity under dilution tests, excluding the first dilution and considering the 1:2 dilution as the starting point

Overall, these results show a discrete precision of the ELISA method used in this study. The value obtained from the "low pool" is excellent, while the CV obtained from the "high pool", although higher than 5%, considered as the limit of acceptability of many of the analytes commonly used in clinical biochemistry, for the ELISA test can be considered reliable (Westgard, 2003). Linearity under dilution test showed poor reliability at high concentration of aldosterone whereas with lower concentration (at least below 300 pg/mL) results can be considered valid.

Population of cats

The 57 samples included in this study were collected by 35 cats (16 at risk and19 with CKD at the time of inclusion). The list of cats, staging and aldosterone concentration are shown in Table 51 whereas data of samples of the same cats collected after 6-12 are shown in table 52.

The majority of them were domestic shorthair cats and 16 (45.7%) were males (1 intact male) and 19 (54.3%) females (all castrated).

At the time of inclusion, cats aged between 7 and 20 years (mean 12.5 years). The 34%, the 46% and the 20% of them aged between 5-10, 11-15 and 15-20 years, respectively. The mean age in the “At risk” group and in the CKD group was 10.8 and 12.3 years, respectively. IRIS staging and aldosterone concentration of cats included in this study are shown in Table 51, whereas in Table 52 IRIS staging and aldosterone concentration of cats followed for 6-12 months.

Table 51 Cats included in the study with breed, age (years), serum creatinine (Crea, mg/dL), IRIS classification based on creatinemia (stage Cr), UPC value, IRIS sub-staging based on proteinuria (stage UPC), systolic blood pressure (SBP, mmHg), IRIS sub-staging based on SBP (stage Pr) and aldosterone concentration (pg/mL)

N°	Breed	Age	Crea	Stage Cr.	UPC	Stage UPC	SBP	Stage Pr.	Aldost.
1	Shorthair	13	2.21	2	0.05	NP	nv	nv	12.2
2	Shorthair	16	1.08	A	0.27	BP	120	AP0	1683.8
3	Shorthair	10	1.04	A	0.07	NP	130	AP0	55.4
4	Shorthair	8	1.52	A	0.11	NP	nv	nv	43.5
5	Shorthair	20	1.87	2	0.08	NP	230	AP3	5402.1
6	Shorthair	12	1.5	1	0.04	NP	130	AP0	68.3
7	Shorthair	13	1.39	A	0.20	BP	120	AP0	128.8
8	Siberian	9	1.67	2	0.30	BP	nv	nv	52.7
9	Siberian	8	1.97	2	0.10	NP	AP0	AP0	117.3
10	Shorthair	14	2.59	2	0.15	NP	160	AP2	18
11	Shorthair	11	0.79	A	0.24	BP	160	AP2	111.9
12	Shorthair	8	1.51	1	0.09	NP	200	AP3	148.3
13	Shorthair	19	6.67	4	0.66	P	130	AP0	1229.9
14	Shorthair	12	1.04	A	0.06	NP	170	AP2	300
15	Siamese	12	1.81	2	0.09	NP	nv	nv	86.2
16	Shorthair	18	2.88	3	0.39	BP	160	AP2	42
17	Norwegian	10	1.97	2	0.06	NP	140	AP0	37
18	Shorthair	8	2.48	2	0.78	P	145	AP0	664.3
19	Exotic S.	14	1.22	1	0.34	BP	130	AP0	168.3
20	Siamese	18	1.96	2	0.26	BP	nv	nv	8.4
21	Shorthair	15	1.9	2	0.16	NP	160	AP2	46.7
22	Shorthair	10	4.44	2	0.10	NP	145	AP0	90.4
23	Shorthair	10	1.64	A	0.25	BP	120	AP0	223.8
24	Shorthair	13	1.36	A	0.26	BP	130	AP0	30
25	Shorthair	15	1.99	2	0.18	NP	140	AP0	163.7
26	Shorthair	14	1.54	A	0.05	NP	140	AP0	22.6
27	Chartreux	7	2.14	2	0.07	NP	120	AP0	101.6
28	Maine Coon	7	1.44	A	0.04	NP	130	AP0	100.9
29	Persian	8	1.48	A	0.06	NP	nv	nv	924.8
30	Shorthair	8	1.02	A	0.30	NP	nv	nv	63.6
31	Shorthair	8	0.91	A	0.07	NP	120	AP0	6.2
32	Shorthair	11	1.28	A	0.14	BP	130	AP0	178
33	Shorthair	14	1.29	A	0.16	NP	nv	nv	269.5
34	Shorthair	13	1.16	A	0.16	NP	nv	nv	608.4
35	Shorthair	19	3.19	3	0.06	NP	130	AP0	253.4

nv: not available; A: at risk; NP: non-proteinuric; BP: borderline proteinuric; P: proteinuric; nf: no follow-up; AP0: systolic blood pressure (SBP) <150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP=160-179 mmHg; AP3: SBP >180 mmHg

Table 52 Cats included in the study with breed, age (years), serum creatinine (Crea, mg/dL), IRIS classification based on creatinemia (stage Cr), UPC value, IRIS sub-staging based on proteinuria (stage UPC), systolic blood pressure (SBP, mmHg), IRIS sub-staging based on SBP (stage Pr) and aldosterone concentration (pg/mL)

N°	Breed	Age	Crea	Stage Cr.	UPC	Stage UPC	SBP	Stage Pr	Aldost.
1	Shorthair	13	2.04	2	0.12	NP	nv	nv	76.4
2	Shorthair	16	1.17	A	0.20	BP	120	AP0	3940.6
6	Shorthair	12	1.62	2	0.15	NP	130	AP0	71.7
7	Shorthair	13	1.64	2	0.32	BP	nv	nv	90.7
8	Siberian	9	1.99	2	nv	nv	nv	nv	100.5
9	Siberian	8	3.45	3	0.04	NP	140	AP0	831.4
11	Shorthair	11	1.28	1	0.11	NP	nv	nv	139.5
14	Shorthair	12	1.39	A	0.06	NP	200	AP3	396.6
16	Shorthair	18	5.31	4	0.16	NP	160	AP2	33.1
19	Exotic S.	14	0.8	A	0.19	NP	150	AP1(wch)	2073
23	Shorthair	15	2.53	2	0.69	P	110	AP0	114.9
24	Shorthair	10	1.58	A	0.04	NP	140	AP0	137.2
25	Shorthair	10	1.4	A	0.09	NP	130	AP0	71.5
26	Shorthair	13	1.8	2	0.18	NP	190	AP3	29
27	Shorthair	15	2.55	2	0.36	BP	125	AP0	84.2
29	Maine Coon	7	1.5	A	0.01	NP	110	AP0	93.3
30	Persian	7	1.73	2	0.03	NP	nv	nv	409.9
31	Shorthair	8	1.03	A	0.24	BP	140	AP0	243.6
32	Shorthair	8	0.78	A	0.06	NP	110	AP0	33
33	Shorthair	8	1.07	A	nv	nv	130	AP0	124.1
34	Shorthair	11	1.37	A	0.17	NP	nv	nv	1298.5
35	Shorthair	13	3.86	3	0.57	P	nv	nv	196.1

nv: not available; A: at risk; NP: non-proteinuric; BP: borderline proteinuric; P: proteinuric; nf: no follow-up; AP0: systolic blood pressure (SBP) <150 mmHg; AP1: SBP= 150-159 mmHg; AP2: SBP=160-179 mmHg; AP3: SBP >180 mmHg; wch: whithe-coat hypertension

Comparisons based on serum creatinine

Grouping all samples (irrespective of time of collection) according to the presence of CKD, there was no significantly difference for urinary aldosterone concentration (P=0.192) between At risk group and CKD group (Figure 48 and Table 55).

Table 53 Descriptive statistics of the groups At risk and CKD

	At risk (n=26)	CKD (n=13)
Minimum	6,2	8,4
25% Percentile	61,5	46,7
Median	133,0	90,7
75% Percentile	449,6	168,3
Maximum	3941,0	5402,0

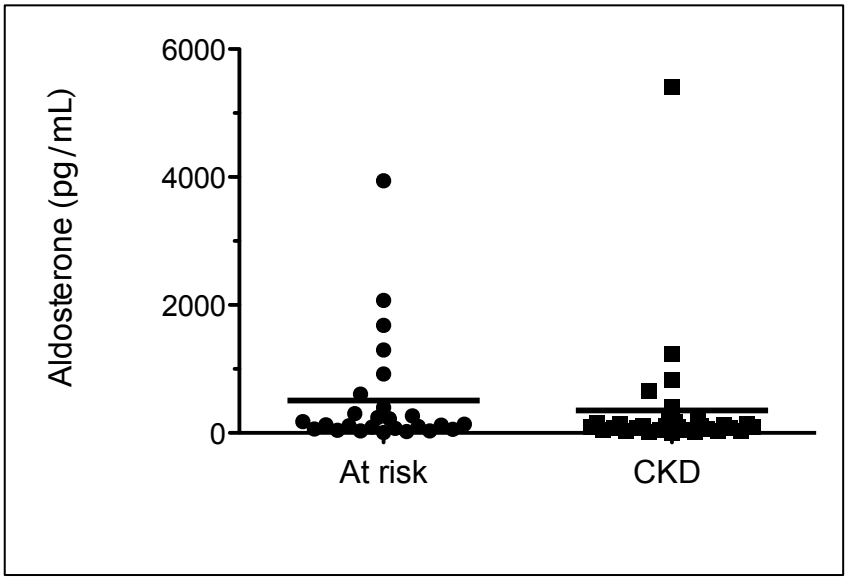


Figure 48 Graphical distribution of aldosterone concentration in cats At risk and cats with CKD

Using samples collected at the time of enrollment in the study, no significant difference was found for the concentration of aldosterone (P = 0.632) comparing the at risk cats with CKD-affected cats. The results obtained are shown in Table 54 and graphically summarized in Figure 49.

Table 54 Descriptive statistics of the cats At risk and cats with CKD using samples collected at the time of enrollment

	At risk (n=16)	CKD (n=19)
Minimum	6,2	12,2
25% Percentile	55,4	49,7
Median	111,9	101,6
75% Percentile	300,0	210,9
Maximum	1684,0	5402,0

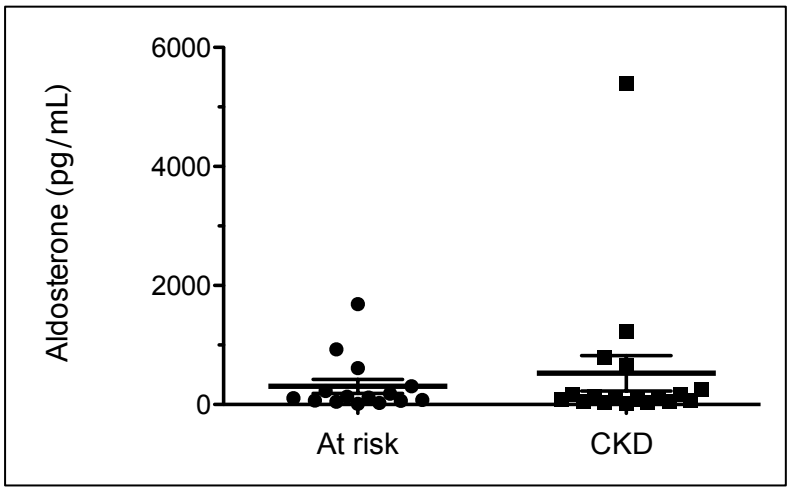


Figure 49 Graphical distribution of aldosterone concentration in cats At risk and cats with CKD using samples collected at time of inclusion

These data suggested that the presence of nephropathy may not induce significant changes in the concentration of aldosterone in the cat. This hypothesis will be verified subsequently in statistical control between IRIS stage groups, where it will be assessed whether the distribution is also dependent on the severity of nephropathy.

It is important to underline that some subjects included in “At risk” group showed signs of nephropathy in the months following the enrollment in the study (6 to 12 months later). Therefore, it was considered useful to repeat the analysis in group of samples collected at T0 and exclude the cats whose evolution after the collection was unclear (e.g. by refusing the owner to participate in the monitoring). The aim of this analysis was to include in the "At Risk" group only the actually healthy subjects and perform a better comparison with the CKD cats. Also in this case, the statistical comparison between the two groups did not show any significant difference (P = 0.598). The descriptive statistics of the "At risk" group and the graphical view of the new comparison are shown in Table 55 and Figure 50, respectively.

Table 55 Descriptive statistics of the cats At risk collected at the time of enrollment excluding the cats whose evolution after the collection was unclear.

	At risk (n=11)
Minimum	6,2
25% Percentile	38,3
Median	86,2
75% Percentile	189,5
Maximum	1684,0

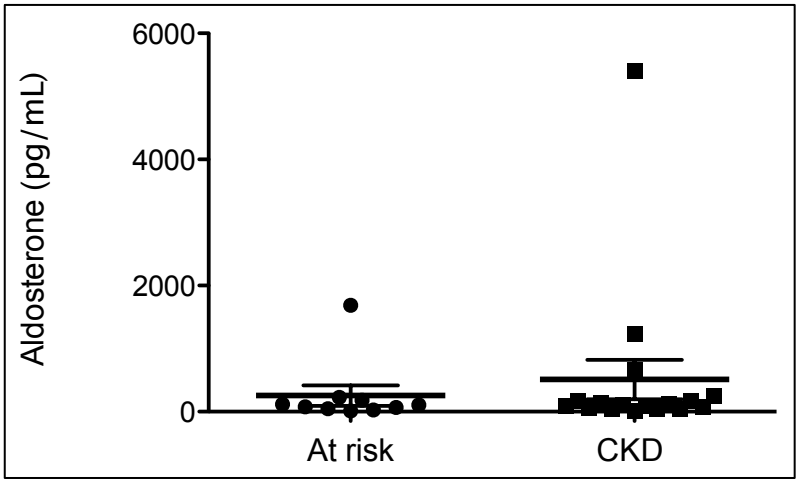


Figure 50 Graphical distribution of aldosterone concentration in cats At risk and cats with CKD using samples collected at time of inclusion and the cats whose evolution after the collection was unclear

With this latter analysis, although the mean and median of the "At risk" group have been reduced, the distribution remains still influenced by an "outlier", corresponding to Cat No. 2, having aldosterone concentration of 1683.8 pg/mL. In a recent study, the median value obtained in healthy non-hypertensive cats was 45.4 pg/mL (interquartile range: 19.6-65.0) (Jepson et al., 2014). The concentrations obtained in our clinically healthy cats seemed to be slightly higher and could justify the absence of significance among the two groups in our study. However, a different method has been used in the study of Jepson and collaborators (i.e. radio-immunoassay) and intrinsic analytical differences could justify this difference, which can only be investigated performing a method comparison study between the two methods.

The reason for the presence of high level of aldosterone in one cat in the “at risk” group is difficult to explain. The same subject produced a similarly high aldosterone value in the

second sampling after 12 months, therefore it is unlikely that this result is an analytical outlier but it is probable that aldosterone was actually high. In veterinary literature, high aldosterone values were recorded in primary hyperaldosteronism. Cats with primary hyperaldosteronism are generally adults or elderly, and clinical examination shows weakness, cervical ventroflexion and dysphagia (Schulman, 2010). Although this cat did not show any particular clinical symptoms at the time of sampling and the other laboratory parameters were normal and not compatible with hyperaldosteronism, it was not possible to exclude the presence of an early stage of this disease, which is normally rare in the cat.
In addition, previous studies reported the influence of diet on aldosterone concentration. Cats fed with a poor sodium diet tend to have aldosterone values higher than those taking normal sodium and in a previous study it was shown that the increase could be of 30-fold (Yu and Morris, 1998). In our study, the diet was not considered but it is interesting to note that the sodium level was low at the time of sampling collection (data not shown). Despite that, in subsequent controls (with high aldosterone concentration still present) sodium returned within the reference range. Therefore, it is not possible to exclude that the diet may have contributed to the variability of serum aldosterone concentration but it is considered unlikely.

Considering the CKD group (both for samples at T0 and irrespective of collection time), aldosterone concentration had a homogeneous distribution of values with the exception of a subject (Cat No. 5) with a significantly higher concentration than the other cats under study. This cat was classified as IRIS 2 stage and the SBP was very high (230 mmHg). However, no follow-up data was available and white-coat hypertension could not be excluded.
These values are similar to those obtained in the recent study mentioned above, where the median values of 84.1 pg/mL (interquartile range: 33.6-137.8 pg/mL) was found in nephropatic and normotensive cats, and the median values of 149.8 pg / mL (interquartile range: 103.1-228.7 pg/mL) was found in nephropatic and hypertensive cats (Jepson et al., 2014).

Cats classified according to the IRIS stages on serum creatinine, showed no significant difference (P = 0.238) between groups, including samples collected at different times in the analysis. Results are shown in Table 56 and graphically summarized in Figure 51. In this analysis the cats in IRIS stage 3 and 4 were few in number (2 IRIS 3 cats and 1 cat in

IRIS 4 stage), therefore, we grouped them in a single group (IRIS 3-4 group) in order to be dimensionally comparable to other groups.

Table 56 Descriptive statistics of the cats grouped according to IRIS staging for serum creatinine. Results of cats at IRIS stage 3 and 4 are joined.

	At risk (n=26)	IRIS 1 (n=4)	IRIS 2 (n=21)	IRIS 3-4 (n=6)
Minimum	6,2	68,3	8,4	33,1
25% Percentile	61,5	86,1	41,8	39,8
Median	133,0	143,9	86,2	224,8
75% Percentile	449,6	163,3	116,1	931,0
Maximum	3941,0	168,3	5402,0	1230,0

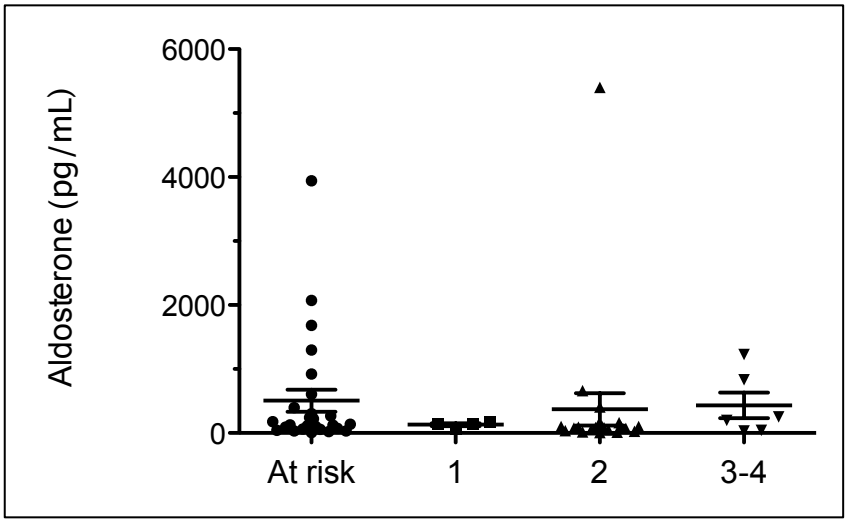


Figure 51 Graphical distribution of aldosterone concentration in cats grouped according to IRIS staging based on serum creatinine (cats at IRIS stage 3 and 4 are joined)

It is possible to note a tendency of aldosterone to have greater concentrations in the advanced stages of CKD (IRIS 3 and 4 stages), although the higher median in IRIS 2 is attributable to the presence of single cases with a significantly higher concentration in one cat and few number of samples were included in IRIS 3-4.

In this study, the correlation between creatinine and aldosterone (Figure 19) was not statistically significant (P = 0.166, r= -0.14), confirming the absence of effect of nephropathy on the concentration of aldosterone.

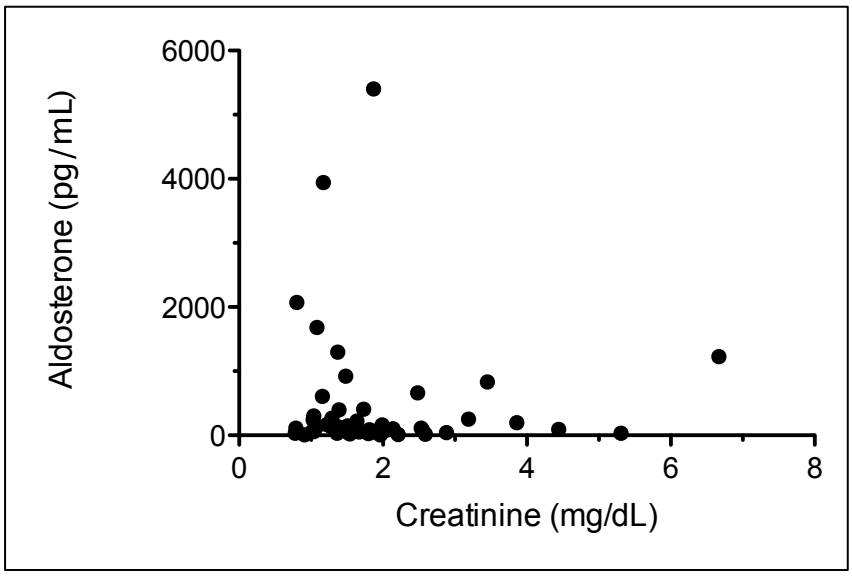


Figure 52 Graphical representation of the correlation between serum creatinine and aldosterone

In summary, the absence of correlation between serum creatinine and aldosterone and the absence of significant differences in aldosterone levels between "A" risk and CKD suggested that, at least in our population of study, the serum aldosterone concentration is not influenced by the presence of nephropathy.

Comparison based on the progression of CKD

We verified whether the few available data on cats follow-up included in the study could be useful in identifying a possible role of aldosterone as an early marker of renal disease.
To this aim, cats at risk were subdivided into groups depending on whether they had developed or not renal failure in 6-12 months after the first sample collection.
This comparison, shown in Table 57 and graphically represented in Figure 53, did not reveal a significant difference between the groups (P = 0.330).

Table 57 Descriptive statistics of cats at risk that remained stable or progressive over time (6-12 months)

	Stable (n=8)	Progressive (n=4)
Minimum	6,2	55,4
25% Percentile	33,4	73,7
Median	82,2	214,4
75% Percentile	161,5	768,6
Maximum	223,8	924,8

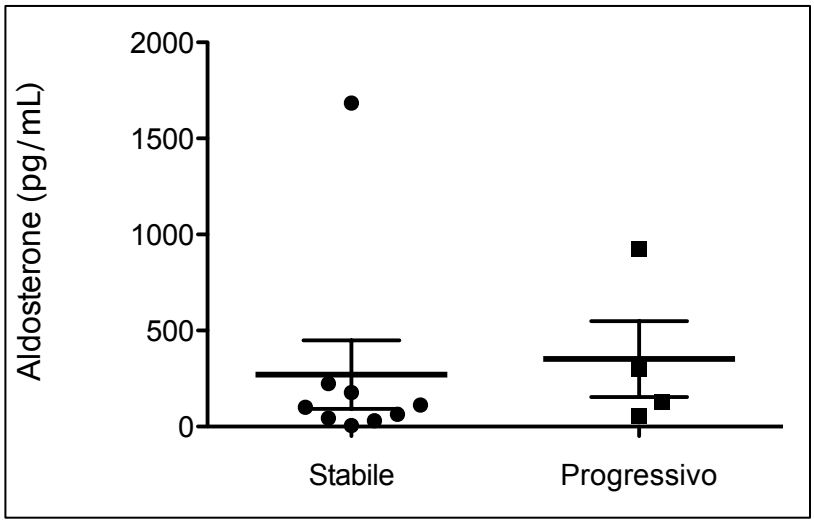


Figure 53 Graphical distribution of aldosterone values obtained at time of inclusion in cats at risk that remained stable or progressive over time (6-12 months)

Moreover, we verified the progression of aldosterone concentration in those subjects where a second sampling was obtained. Specifically, values obtained in zero time and after 6 months or 12 months were compared.

A first analysis was performed with subjects that were classified as At Risk at the time of enrolment. The results showed no significant differences (P = 0.547) as shown in Table 58 and Figure 53. Figure 54 shows the same results but focusing on aldosterone values below 300 pg/dL.

Table 58 Descriptive statistics of results obtained at time zero (T0) and after 6 or 12 months (T6-12) in cats at risk

	T0 (n=8)	T6-T12 (n=8)
Minimum	6,2	8,0
25% Percentile	38,4	76,9
Median	106,4	130,7
75% Percentile	212,4	217,6
Maximum	1684,0	4575,0

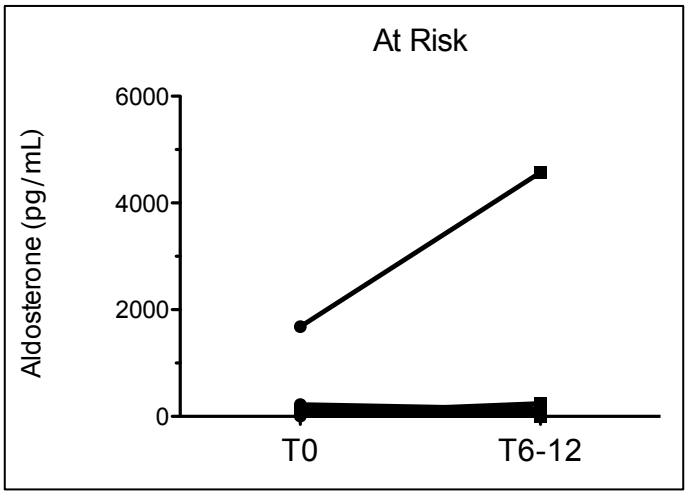


Figure 54 : Graphical distribution of aldosterone values obtained at time zero (T0) and after 6 or 12 months (T6-12) in cats at risk

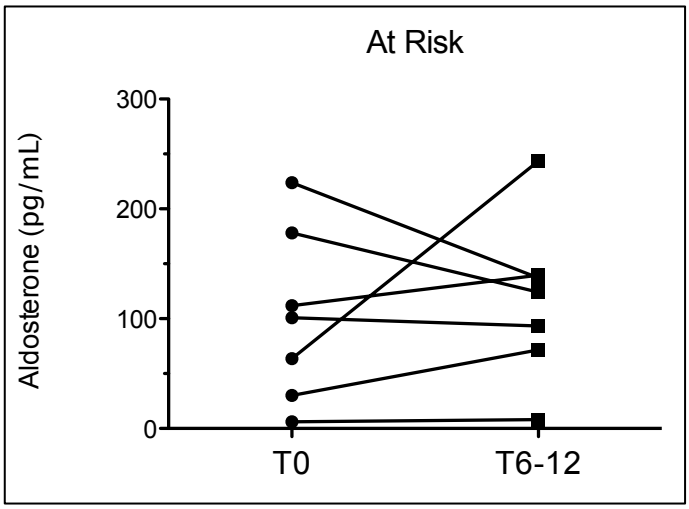


Figure 55 Graphical distribution of aldosterone values obtained at time zero (T0) and after 6 or 12 months (T6-12) in cats at risk (focused on aldosterone <300 pg/ml)

In this group of subjects there was a cat (No. 2) that has developed nephropathy in 12 months. Excluding this subject, the absence of statistical difference between the two times (P = 0.937) remains and the findings are described in Table 59 and shown graphically in Figure 56, focusing on aldosterone charts minus 300 pg/dL

Table 59 Descriptive statistics of results obtained at time zero (T0) and after 6 or 12 months (T6-12) in cats at risk after exclusion of subject No. 2

	T0 (n=7)	T6-T12 (n=7)
Minimum	6,2	8,0
25% Percentile	30,0	71,5
Median	111,9	124,1
75% Percentile	223,8	139,5
Maximum	1684,0	4575,0

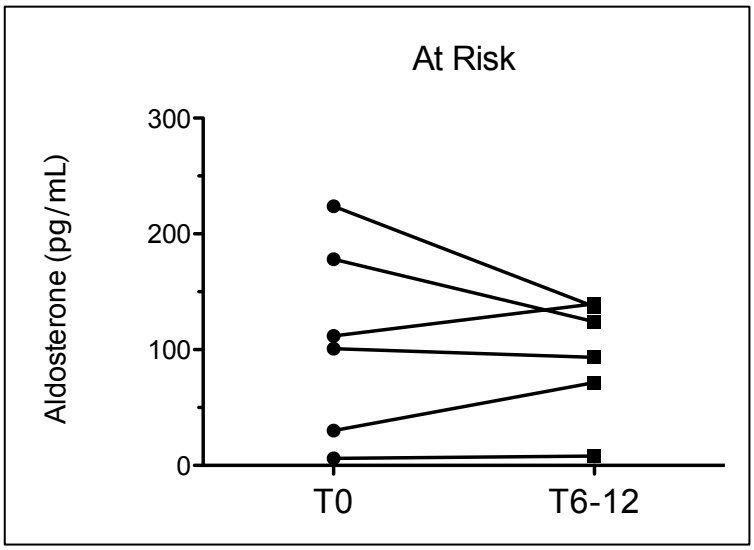


Figure 56 Graphical distribution of aldosterone values obtained at time zero (T0) and after 6 or 12 months (T6-12) in cats at risk after exclusion of subject No. 2 (focused on aldosterone <300 pg/ml)

The trend of aldosterone concentrations that can be appreciated by observation of this last graph could be considered an estimate of biological variability in healthy cats. However, with the data available, it is not possible to calculate a coefficient of variation applicable to other future studies since evaluation of the biological variability was not one of the objectives of this study and further studies with more specific study design and criteria of inclusion are needed to evaluate this type of variability.

The subjects who were classified as CKD at the time of enrolment were also examined. There was no significant difference (P = 0.966) between the concentration of aldosterone measured at inclusion and after 6-12 months, as shown in Table 60 and in Figure 57.

Table 60 Descriptive statistics of results obtained at time zero (T0) and after 6 or 12 months (T6-12) in cats with CKD

	T0 (n=11)	T6-T12 (n=11)
Minimum	12,2	29,0
25% Percentile	52,7	76,4
Median	128,8	100,5
75% Percentile	300,0	409,9
Maximum	924,8	831,4

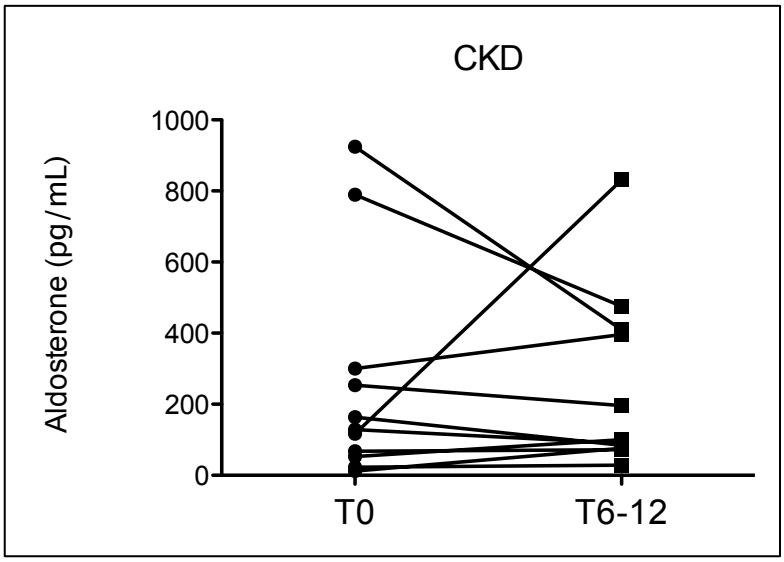


Figure 57 Graphical distribution of aldosterone values obtained at time zero (T0) and after 6 or 12 months (T6-12) in cats with CKD

Also in this group there was a subject (n ° 9) who, in 12 months, experimented an worsening of the disease, moving to a subsequent IRIS stage (from Stage 2 to Stage 3). Excluding this subject, there is no statistical difference between the two times (P = 0.556) and the results are described in Table 61 and shown graphically in Figure 58.

Table 61 Descriptive statistics of results obtained at time zero (T0) and after 6 or 12 months (T6-12) in cats with CKD after exclusion of subject No. 9

	T0 (n=10)	T6-T12 (n=10)
Minimum	12,2	29,0
25% Percentile	45,2	75,2
Median	146,3	95,6
75% Percentile	422,4	399,9
Maximum	924,8	475,0

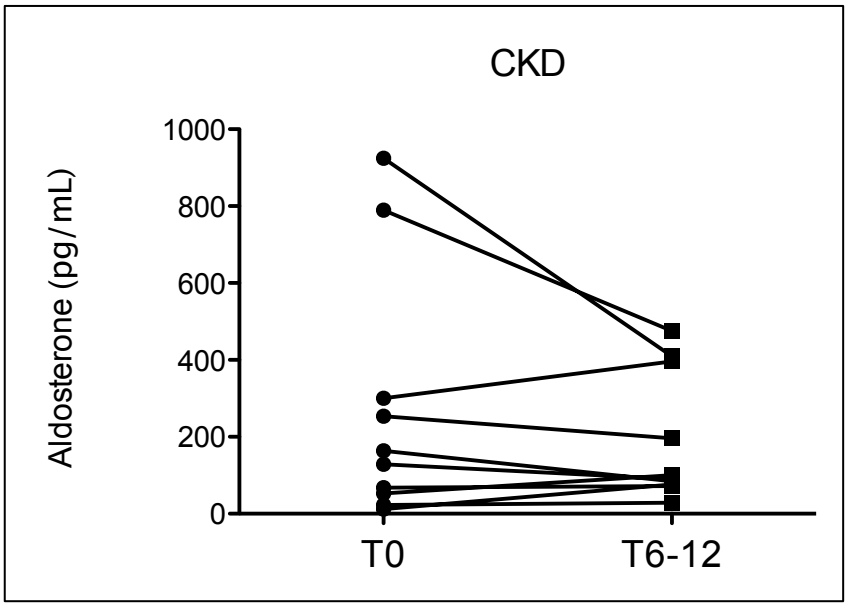


Figure 58 Graphical distribution of aldosterone values obtained at time zero (T0) and after 6 or 12 months (T6-12) in cats with CKD after exclusion of subject No. 9

Comparing the graph in Figure 55 with the one in Figure 56, and the chart in Figure 57 and Figure 58, it is worth to note that the only two cases with worsening of health status (one for the population at risk, moving from " At risk" to Stage 2 and one for CKD group, moving from Stage 2 to 3) showed a marked increase in aldosterone concentration over time, with greater variation compared with the other stable cases. Based on these two individual cases it is not possible to provide definitive conclusions, however it is likely that these increases over time in cats with active nephropathy are consistent with what was observed in the previous analysis, where, although not significant, a tendency to have higher aldosterone concentration in subjects with more severe CKD was found.

It is also not possible to exclude analytical outliers that could be affected this specific analysis and could be avoided by double measurements. Therefore, more assessments are needed with more case with progressive disease to further evaluate these preliminary results.

Comparisons based on SBP

Aldosterone was not significant different grouping samples according to the presence of CKD and HT (P = 0.142) (using a cut-off of 150 mmHg) as shown in Table 62 and shown in Figure 59.

Table 62 Descriptive statistics of the different groups divided according to systolic blood pressure (SBP) and presence of CKD.

	At risk NT (n=22)	At risk NT (n=4)	CKD HT (n=14)	CKD HT (n=7)
Minimum	6,2	111,9	37,0	18,0
25% Percentile	52,4	158,9	81,1	29,0
Median	126,5	348,3	116,1	42,0
75% Percentile	354,2	1654,0	356,1	148,3
Maximum	3941,0	2073,0	1230,0	5402,0

NT: normotensive (SBP<150 mmHg); HT: hypertensive (SBP>150 mmHg)

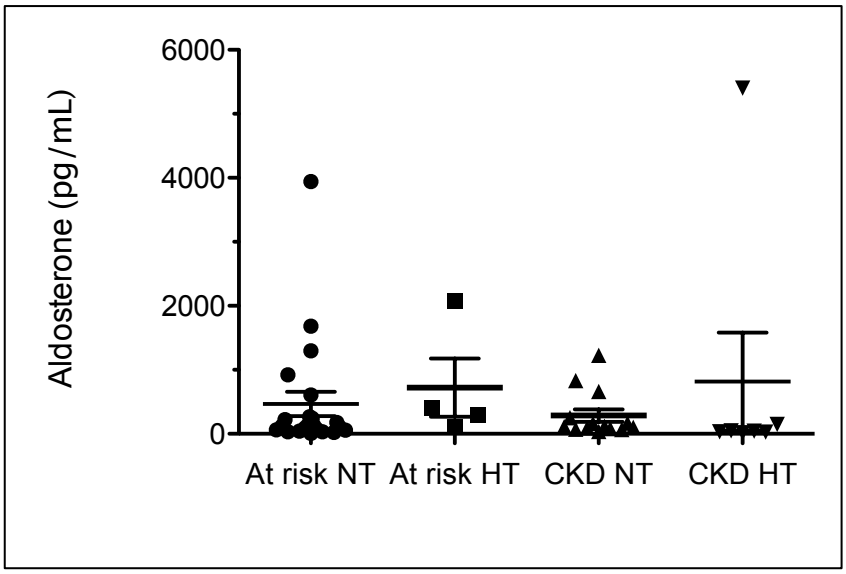


Figure 59 Graphical distribution of aldosterone in cats grouped according to the health status (At risk vs CKD) and the presence of hypertension (lower or higher 150 mmHg). NT: normotensive; HT: hypertensive

Such results suggest the absence of any relationship between hypertension and serum aldosterone in the cat. As a further confirmation of this hypothesis, no significant correlations (P = 0.213, r = 0.168) between systolic pressure values and aldosterone levels were found (Figure 60).

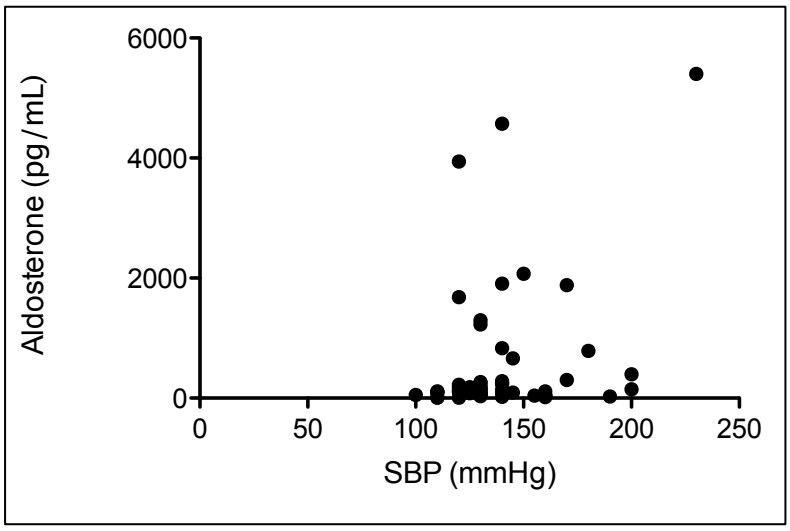


Figure 60 Graphic representation of the correlation between systolic blood pressure (SBP) values and aldosterone

One of the goals of this study was to use aldosterone to differentiate white-coat hypertension from true hypertension. According to results of our population, this biomarker do not have any utility for such aim.

In a recent study, it is reported that in some hypertensive cats ACE inhibitors did not cause aldosterone (and renin) changes after the onset of therapy. This goes to support the fact that hypertension in the nephropathic cat might not be directly (or completely) caused by the activation of the RAAS system (Jepson et al., 2014), and the results of our study seem to be in accordance with this hypothesis.

Comparisons based on Proteinuria

Aldosterone did not significantly differed (P=0.227) between samples classified according to IRIS sub staging of proteinuria (figure 61). In table 63 the descriptive statistic is shown.

Table 63 Descriptive statistics of the different groups divided according to proteinuria.

	NP (n = 37)	BP (n = 14)	P (n = 4)
Minimum	6,2	8,4	114,9
25% Percentile	45,1	50,1	135,2
Median	93,3	120,4	430,2
75% Percentile	284,8	228,8	1089,0
Maximum	5402,0	3941,0	1230,0

NP: non-proteinuric; BP: borderline proteinuric; P: proteinuric

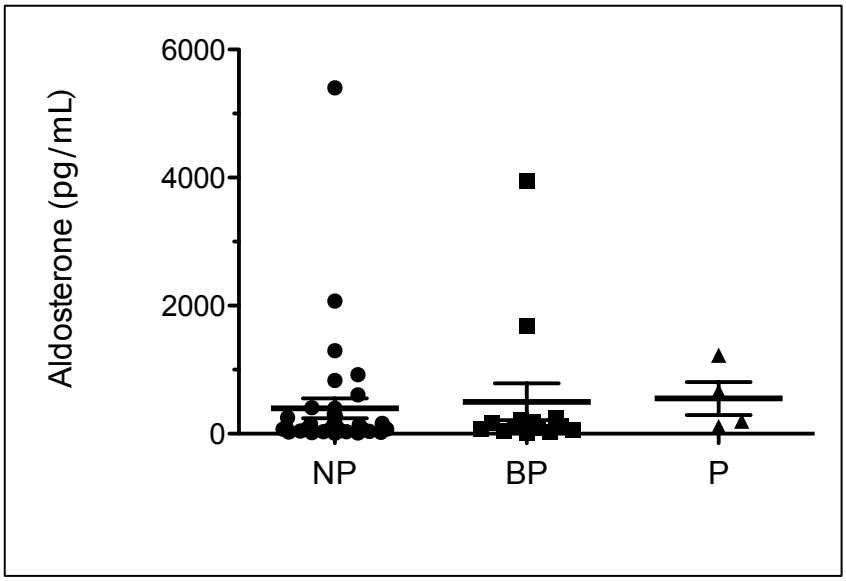


Figure 61 Graphical distribution of aldosterone cats grouped according to IRIS staging for proteinuria (NP: non-proteinuric; BP: borderline proteinuric; P: proteinuric)

No significant correlation (P=0.533) was found between UPC and aldosterone (Figure 62)

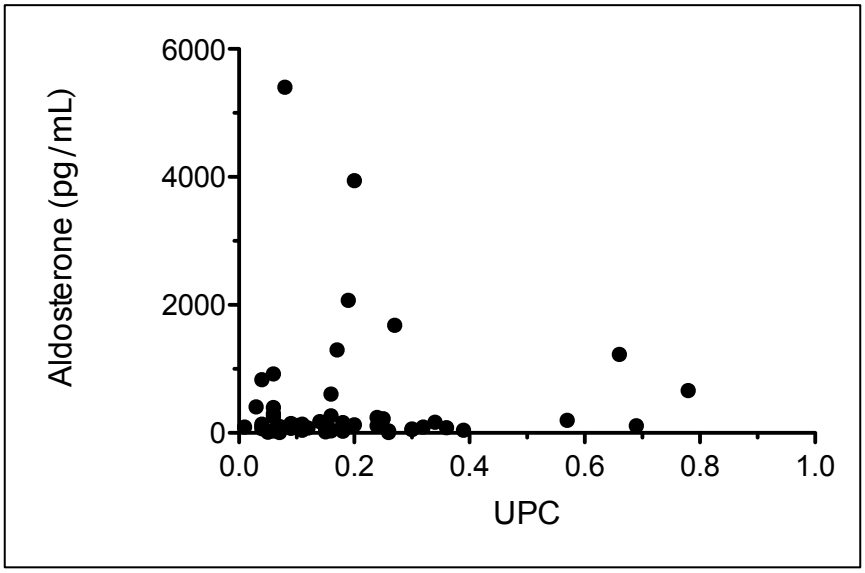


Figure 62 Graphic representation of the correlation between urinary protein-to-creatinine (UPC) ratio and aldosterone

These results, again, confirm that the pathogenicity of proteinuria in cat is different from that of other species, in particular to the dog where this alteration is mainly depends on glomerulopathy, which not only results in loss of proteins with the urine, but also triggers a pathophysiologic mechanism that induces RAAS activation and hypertension. The kidney disease in the cat has a prevalent tubule-interstitial component and this makes the proteinuria not particularly frequent and severe as demonstrated in the case study

4.4 Urinary alpha-1 microglobulin

Introduction and aims

Feline CKD is mainly characterized by interstitial inflammation and tubular injury (McLeland et al., 2015). Several markers of tubular damage have been evaluated urine of cats with CKD. N-acetyl-beta-D-glucosaminidase (NAG) index (Sato et al., 2002) and retinol-bonding protein (RBP) (van Hoek et al., 2008; van Hoek et al., 2009; Jepson et al., 2009) have been correlated to the development of azotemia and tubular damage, respectively; lower levels of uromodulin (Ferlizza et al., 2015) and cauxin (Miyazaki et al., 2007; Jepason et al., 2010; Ferlizza et al., 2015) and higher cystatin C (Ghys et al., 2014) were also demonstrated in cats with CKD. Alpha-1 micorglobulin (A1MG) is a protein that is produced by the liver as alpha-1-microglobulin/bikunin precursor (AMBP) and, due to the low molecular weight (that is 26.000 Da), is able to freely pass the glomerulus (Akerström et al., 2000).

In people, urinary A1MG is a valuable tool to estimate tubulointerstitial damage and it may precede albumin excretion (Penders and Delanghe, 2004). It was shown that high urinary A1MG have predictive value for both remission and progression of certain types of CKD (Pfleiderer et al., 1992; Bazzi et al., 2001; Shore et al., 2010; Robles et al., 2013)

Also in dog A1MG was shown to increase as the CKD progress (Vinge et al., 2010) bu there are no studies evaluating A1MG in cats with CKD

The aim of this study was to validate a commercially available ELISA kit for quantification of A1MG in feline urine by investigating intra-assay variability, linearity under dilution, spiking recovery and to evaluate this biomarker in a group of cats with or without tubular damage and CKD

Material and methods

Selection of the samples

Thirty-four urine samples belonging to cats were included. These patients were selected among the cats evaluated with sodium dodecyl sulfate-agarose gel electrophoresis (SDS-AGE) study described above.

The results of SDS-AGE preliminary evaluation were used to identify 2 samples without and one sample with tubular bands (TBs). These samples were deemed to be useful to the validation purpose because the presence of TBs could be theoretically suggestive of

tubular damage and in turn likely suggestive of presence low molecular weight proteins and likely of alpha1-microglobulin (A1M); conversely the absence of such bands could suggest the absence of A1M.

Measurement of alpha1-microglobulin and validation of ELISA kit

The ELISA kit evaluated in this study (Cat α1-Microglobulin ELISA Kit – Mybiosource, San Diego, CA, USA) was a quantitative sandwich ELISA. As stated by the manufacturer, ELISA stripplate wells were coated with purified anti-A1MG antibodies, the detection range was from 3,12 µg/mL to 100 µg/mL and the analytical sensitivity was 1 µg/mL. No information was available regard the target epitopes.

The day before analysis aliquots were gently thawed overnight at +4°C and were warmed at room temperature one hour before the procedures.

Intra-assay variability was assessed by testing one sample with TBs and one sample without TBs 5 times (5 wells each) and both samples were distributed in 2 different strips in order to evaluate also the variability due to pipetting. Mean, standard deviation and coefficient of variation (CV) were calculated.

Linearity under dilution (LUD) test was performed by serially diluting the sample with TBs and one sample without TBs by a twofold dilution scheme (i.e 1:2, 1:4, 1:8 and 1:16) using the ELISA kit diluent. Also two other dilution protocols were evaluated, diluting the sample with TBs 20, 40, 60 and 80% using the ELISA kit diluent and distilled water. Spiking recovery test (SRT) was performed by adding to the samples without TBs the ELISA kit diluent, the standard 1 (3,12 µg/mL), the standard 3 (12,5 µg/mL) and the standard 6 (100 µg/mL) by a 1:10 ratio (90 µL of sample + 10 µL of spike).

In the second session of work, all the remaining samples (32 samples) were than assayed. In each run, the diluent (used as blank solution) and the six standards (ranging from 3,12 µg/mL to 100 µg/mL) provided by the manufacturer were set in duplicate to calibrate the ELISA kit. Plate was washed with an automatic washing instrument (BioRad Microtech, Segrate, Milan, Italy), suctioning and applying the wash buffer solution for a total of 4 consecutive times. Absorbance was measured within 30 minutes by an automatic spectrophotometer reader (Labsystem Multiscan MS, Labsystems Helsinki, Finland). The best calibration curve was obtained by setting the absorbance of calibrators in commercially available software (CurveExpert Professional, 2.3.0). Results were expressed as µg/mL and calculated with the equation of calibration curve using Excel software.

Results obtained by the LUD and SRT tests were evaluated by linear regression analysis. Also, the percentage of recovery of each dilution n both tests was calculated according to the formula:

recovery (%) = mean observed / expected X 100.

For samples results, the ratio between the A1MG concentration (µg/mL) and the urinary creatinine (mg/dL) measured at the time of inclusion ("uA1MG/uC") was calculated in order to normalize the A1MG concentration to the urine dilution.

Statistical analysis

Difference in urinary A1MG concentration and uA1M/uC between the group of cats with TBs and groups of cats without the TBs (identified by SDS-AGE) were calculated with Mann-Whitney test

Results and discussion

Among the 34 samples, 10 had TBs (6 in a mixed pattern and 4 in a tubular pattern).

The sample with and the sample without TBs resulted in a mean ±SD A1MG of 25.67 ±5.31 µg/mL and 34.32 ±7.13 µg/mL, respectively. CVs were 20.7 for the sample with TBs and 20.8 for the sample without.

LUD tests failed to fit linearity in all dilution protocols and with both types of diluent (Figure 63). Specifically, the dilution protocol from 1:2 to 1:16 yielded recovery from 29.8% to 207.2% (mean: 101.4 %) for sample with TBs and from 29.9% to 240.1% (mean: 108.0%) for sample without TBs (table 6664 the dilution protocol from 20% to 80% with ELISA diluent yielded 12.4% to -44.4% recovery (mean: -3.0%) and the dilution protocol from 20% to 80% with bi-distilled water yielded 78.4% to 131.3% recovery (mean: 256.7%) (Table 65).

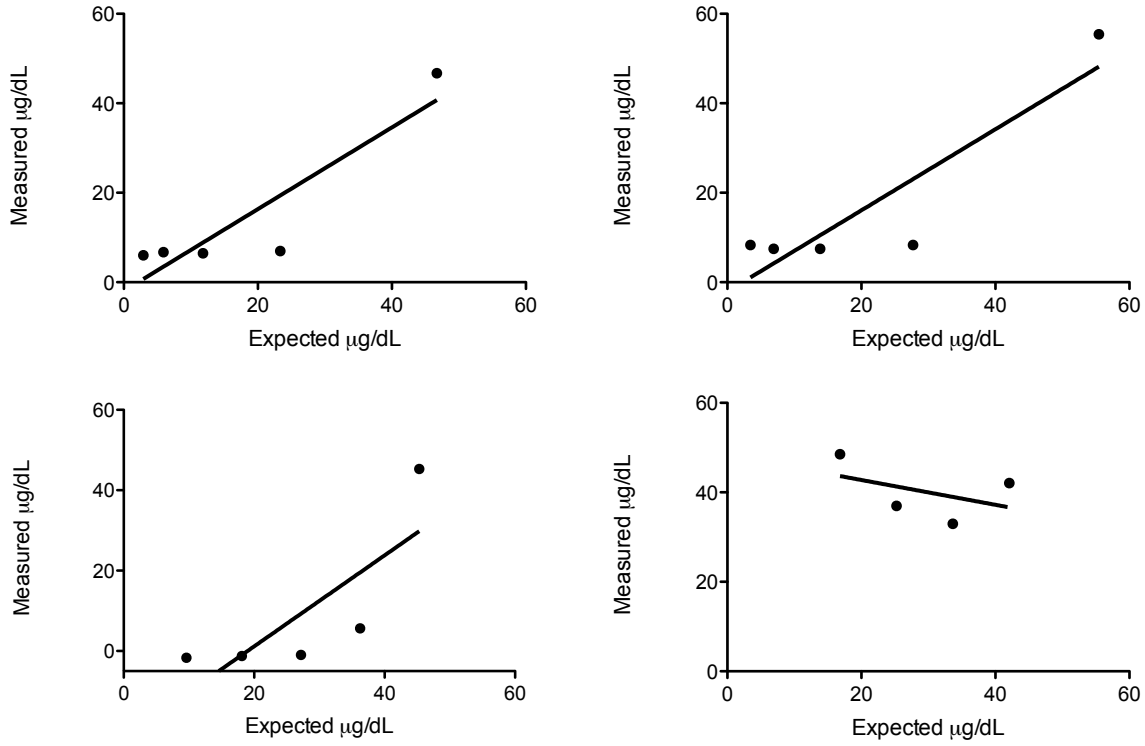


Figure 63 Linearity under dilution results. Top left: the dilution protocol from 1:2 to 1:16 for sample with TBs. Top right: dilution protocol from 1:2 to 1:16 for sample without TBs. Bottom left: dilution protocol from 20% to 80% with ELISA diluent yielded. Bottom right: dilution protocol from 20% to 80% with bi-distilled water yielded

Table 64 Serial dilutions of the samples with tubular bands (TBs) and sample without TBs. Measured values, expected values and recovery rate (O/E recovery) are shown

	TBs			No TBs		
Dilution	Measured (µg/mL)	Expected (µg/mL)	O/E (%)	Measured (µg/mL)	Expected (µg/mL)	O/E (%)
Undiluted	46,70	46,7		55,44	55,44	
1:2	6,96	23,3	29,8	8,31	27,72	29,9
1:4	6,47	11,8	54,8	7,48	13,86	53,9
1:8	6,71	5,9	113,7	7,48	6,93	107,9
1:16	6,01	2,9	207,2	8,31	3,46	240,1

Table 65 Serial dilution of the sample with tubular bands (TB) from 20% to 80% with ELISA diluent and distilled water yielded. Measured values, expected values and recovery rate (O/E recovery) are shown

	ELISA diluent			Distilled water		
Dilution	Measured	Expected	O/E (%)	Measured	Expected	O/E (%)
100%	45,30	45,30		42,08	42,08	
80%	5,62	36,24	15,50	32,98	33,66	97,9
60%	-0,96	27,18	-3,5	36,97	25,24	146,4
40%	-1,23	18,12	-6,7	48,53	16,83	288,3
20%	-1,68	9,6	-17,5	47,43	9,6	494,1

Also SRT showed lack of linearity (Table 66 and Figure 64).

Table 66 Spike recovery test results. Measured and expected values are shown

	Measured	Expected
NoTB + Diluent	18,34	56,40
NoTB + Standard 1	13,74	56,71
NoTB + Standard 3	17,01	57,65
NoTB + Standard 3	18,45	66,40

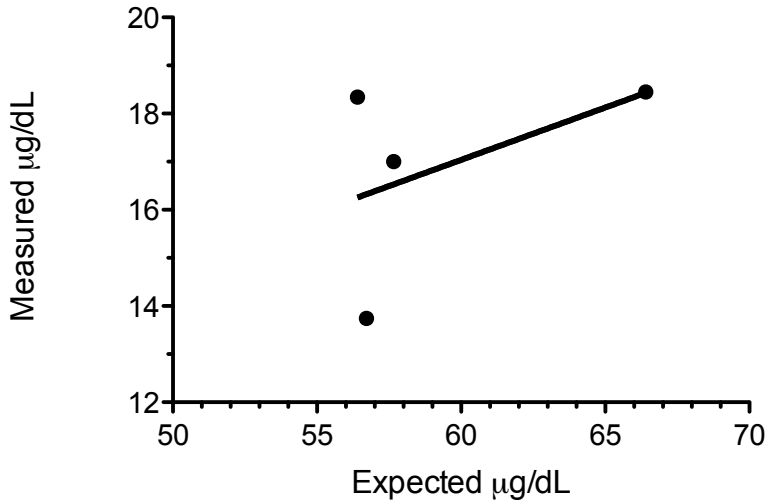


Figure 64 Graphical representation of spiking recovery test.

No significant differences was found between samples with TBs and samples without TBs (Figure 65 and Table 67), nether with urinary A1MG concentration (P=0.164) nor with

A1M/uC (P= 0.622). Given the validation test results, these results were considered unreliable.

Table 67 Descriptive statistics (median and min-max range) of alpha-1 microglobulin (a1MG) and alpha-1 microglobulin-to-creatinine ratio (a1MG/uC) in samples without tubular bands (No TBs) and with tubular bands (TBs)

	a1MG (µg/mL)		a1MG/uC	
Group	Median (µg/mL)	Min-max (µg/mL)	Median	Min-max
No TBs	40.83	27.48-62-67	0.21	0.08-1.54
TBs	35.19	25.57-59-43	0.16	0.06-1.32

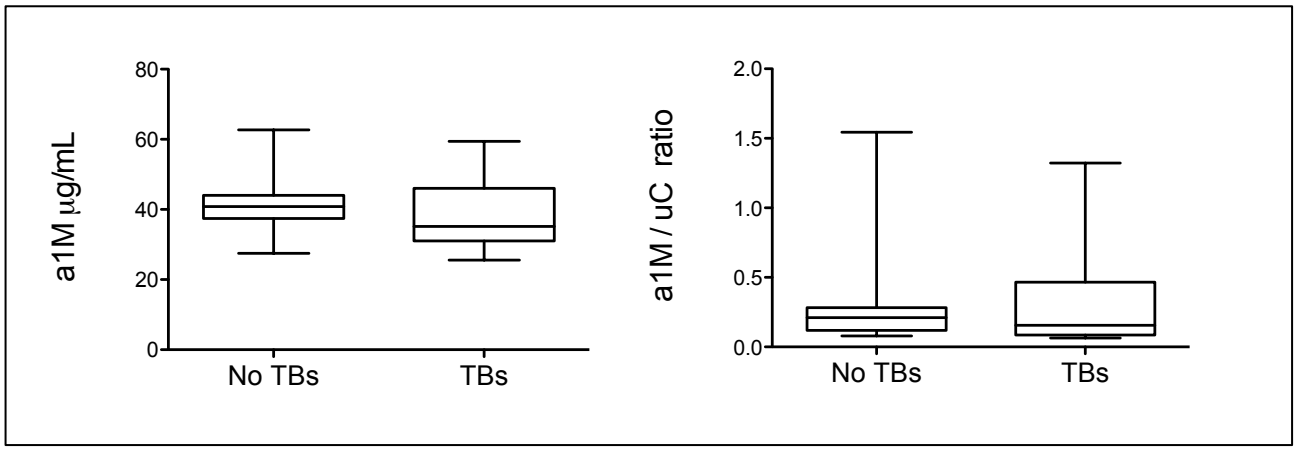


Figure 65 Graphical representation of distribution of alpha-1 microglobulin (left) and alpha-1 microglobulin-to-creatinine ratio (right) in samples without tubular bands (No TBs) and with tubular bands (TBs). Box plots represent the interquartile range (IQR), and the horizontal line represents the median value of each group. Whiskers extend to the minimum and maximum values.

In order to investigate the possible reasons of the failure in all the validation tests, the content of standard of the kit with the higher A1MG concentration (standard n° 6) was assayed with SDS-AGE (see SDS-AGE study below for method description). The standard yielded a strong band with a molecular weight corresponding to that of albumin and two weak bands in the high molecular weight area of the gel but any bands were detected in the low molecular weight area of the gel where A1MG was expected (Figure 66). A band with the MW of A1MG was not detected in our standard. Therefore it was suspected that the A1MG ELISA kit purchased and used in this study was not correctly produced or the specifications declared from the manufacturer did not correspond to the real characteristics.This possibility was demonstrated in an other published study in dogs, where the presence of albumin instead of the right biomarker (procalcitonin) was

demonstrated in the standard of the ELISA kit evaluated by the authors (Floras et al., 2014). However, the evaluation with SDS-AGE could not be sensitive enough to detect A1M, which could be present in a lower concentration than the limit of sensitivity of SDS. Moreover the addition of other proteins in standard solution is allowed in order to mimic the content of the original matrix.

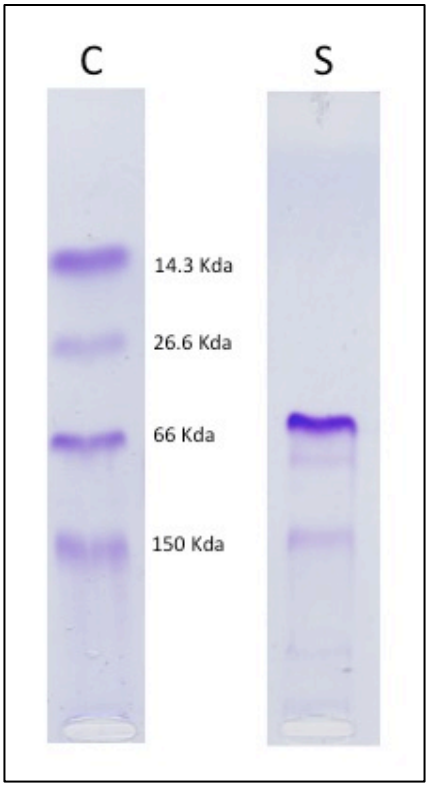


Figure 66 SDS-AGE of control sample ("C", with indication of molecular weight) and the standard 6 (S) of the ELISA kit

Therefore, a preliminary evaluation with Western-blot analysis was performed. The same standard (standard n° 6) was run and the secondary antibody provided with the ELISA kit (rabbit anti-cat A1MG antibody) was used to detect. Than an antibody toward the FC portion of rabbit antibody (human anti-rabbit FC), labeled with fluorescence was used to detect. No visible fluorescent bands were detected on the gel. However, the lack of results from this western-blot analysis could be due to incorrect dilution protocol of the antibodies or also the improper use the antibody provided by the ELISA kit manufacturer in the western-blot method. Therefore, further assays are required to evaluate in depth the analytical performance of the ELISA kit used in this study.

4.5 Urinary SDS-AGE

Introduction and aims

Qualitative evaluation of proteinuria in patient affected by CKD with protein electrophoresis allows identification of specific protein patterns which in turn reflect renal injury at various segment of the nephron. By this method is possible to identify glomerular, tubular or mixed proteinuria. Glomerular proteinuria occurs when the selective permeability of the glomerulus is altered, leading to filtration of proteins with middle molecular weight (MMW, 60–80 kDa) to high molecular weight (HMW; >80 kDa). The presence of only MMW proteins in urine, such as albumin, is defined as “selective” glomerular proteinuria, whereas HMW proteins, such as IgG indicate “nonselective” glomerular proteinuria (Yalcin and Cetin, 2004). Tubular proteinuria is characterized by the presence of low molecular weight (LMW; <60 kDa) proteins which normally freely pass the glomerulus and are reabsorbed in the proximal tubule (Nabity et al., 2011). When both glomerular filtration and tubular reabsorption are disturbed, mixed proteinuria can occur (Zini et al, 2004).

In dogs, good sensitivity of urinary electrophoresis for the identification of tubular damage was shown (Zatelli et al., 2003; Zini et al., 2004; Zaragozza et al., 2003). Specifically when compared to histopathology, sensitivity for both glomerular and tubular damage was 100% and 92%, respectively, whereas the specificity was lower (40-62%) (Zatelli et al., 2003). In addition, the presence of proteins with very low MW (12-15 kDa) was associated with higher grade of tubular damage on histopathology (Zini et al., 2004).

The sodium dodecyl sulphate agar gel electrophoresis (SDS-AGE) is easy and relatively inexpensive electrophoretic method for qualitative urinary protein investigation.

It was recently validated at our institution in dogs (Giori et al., 2011) and valuated cats with suspected amyloidosis (Paltrinieri et al., 2015) but no information is available about the electrophoretic protein patterns in naturally occurring feline CKD. The aim of this study is to describe the electrophoretic patterns of proteinuria in cats with CKD using a commercially available SDS-AGE method.

Materials and methods

70 samples obtained from 39 cats were included in this study. SDS-AGE was performed on urinary supernatants using a commercially available semi-automated analyzer was used (Hydrasis, Sebia Italia SRL, Bagno a Ripoli, Firenze, Italy). Electrophoretic gels are

supplied by the manufacturer (Hydragel 5 Proteinuria, Sebia Italia SRL, Bagno a Ripoli, Firenze, Italy). Five wells were available in each gel and in each run two gels can by load.
Samples were analyzed after a maximum of 12-months storage at -20°C. The day before analysis aliquots were gently thawed overnight at +4°C and were warmed at room temperature one hour before the run.
In each run 9 samples and a control samples were loaded in the two gels. The control material was purchased by the manufacturer (Molecular mass control, Sebia Italia SRL, Bagno a Ripoli, Firenze, Italy) and contained proteins with known molecular weight (lysoxyme, 14.3 had; triosephosphate isomerase, 26.6 kDa; bovine albumin 66 kDa; and human IgG, 150 kDa).
Procedures were performed according to the manufacture's instruction. Specifically, after a brief vortex of the supernatant, each urine sample was mixed with the diluents included in the kit containing SDS and bromophenol blue. The instrument was then prepared by mounting the sponges embedded with the appropriate buffer (pH 8.5) on the electrodes and by placing two gels on the migration chamber after removal of excess buffer.
Five µl of urine diluted as above were placed into wells of the gel and the procedure was started using the specific program (Proteinuria 1*5).
The gel was then transferred to the staining module and automatically stained with acid violet and washed. After that, gels were manually treated with a solution of glycerin (1:8 in distilled water) and dried.
Gels were then scanned with a commercially available scanner and acquired on the computer as JPEG images.
Gels were visually analyzed and electrophoretic bands of the samples were identified comparing with bands of the control sample.
For each sample, one of the following patterns were assigned:

- Negative: no bands.
- Albuminuria: only a band of MW equal to albumin (Figure 67, lane "A").
- Glomerular selective: bands with MW lower than IgG and higher or equal to albumin (Figure 67, lane "GS").
- Glomerular non-selective: bands with MW equal to IgG and higher or equal to albumin (Figure 67, lane "Gns").
- Tubular: bands with MW lower to albumin with or without albumin bans (Figure 67, lane "T").
- Mixed: bands lower and higher than albumin (Figure 67, lane "M").

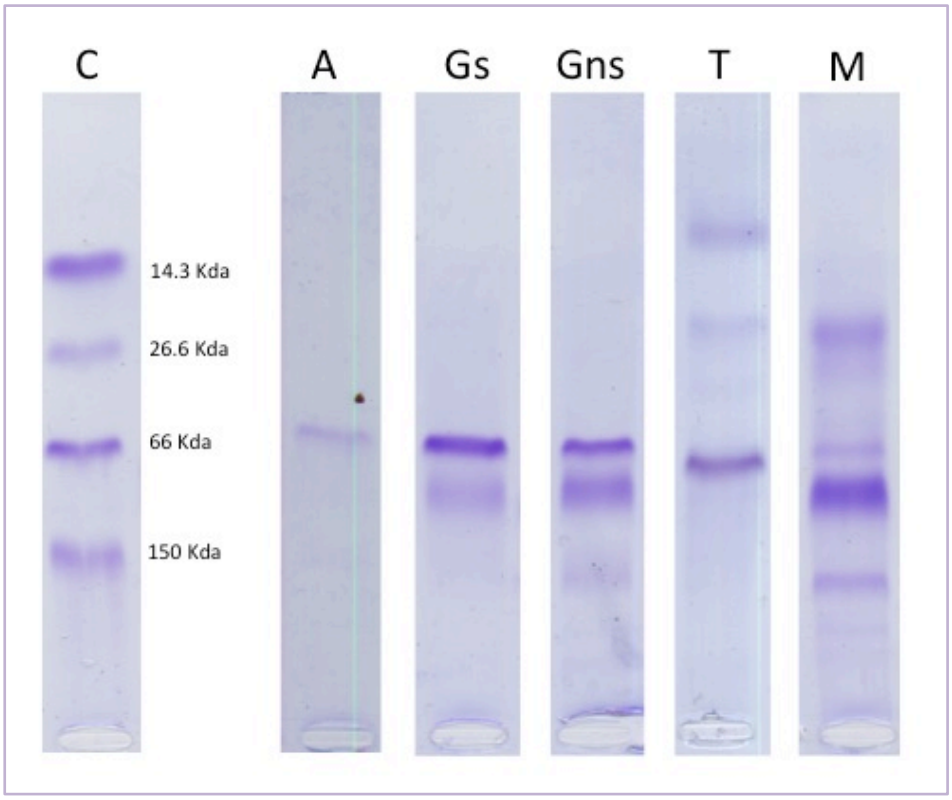


Figure 67 SDS-AGE of urine samples. Lane C: control; lane A: albuminuric; lane Gs: glomerular selective pattern; lane Gns: glomerular non selective pattern; lane T: tubular pattern; lane M: mixed pattern

Statistical analysis

Fisher's exact test was used to verify the null hypothesis that the tubular bands (i.e. tubular or mixed patterns) were equally distributed in samples grouped according to the healthy status (At risk vs CKD in all samples) and grouped according to proteinuria (NP vs BP+P). Commercially available software was used and a P-value of 0.05 was considered significant.

Results

Distribution of IRIS sub-stage for proteinuria in the IRIS staging group (based on serum creatinine) were shown in table 68.

Table 68 Distribution of IRIS sub-stage for proteinuria in the IRIS staging group (based on serum creatinine)

	NP	BP	P	total
At risk	**17**	**9**	**-**	26
IRIS 1	**8**	**4**	**-**	12
IRIS 2	**15**	**3**	**5**	23
IRIS 3	**4**	**1**	**3**	8
IRIS 4	**1**	**1**	**-**	2
total	81	18	8	

NP: non-proteinuric; BP: borderline proteinuric; P: proteinuric

Descriptive statistics of urinary protein, UPC ratio and USG in the different IRIS stages are shown in table 69.

Table 69 Descriptive statistic of urinary protein, UPC ratio and USG in the different IRIS stages. Median (range) are shown. Number of samples are indicated in brackets.

	IRIS 1 (12)	IRIS 2 (23)	IRIS 3 (7)	IRIS 4 (2)
UP	35.7 (5.2-61.4)	28.0 (6.1-171.3)	18.5 (2.8-39.1)	7.7 (4.4-11.0)
UPC	0.13 (0.04-0.34)	0.17 (0.03-0.78)	0.17 (0.02-0.71)	0.20 (0.16-0.24)
USG	1048 (1028-1060)	1034 (1016-1066)	1013 (1008-1025)	1012 (1010-1013)

NP: non-proteinuric; BP: borderline proteinuric; P: proteinuric

In “At risk” group, 3 samples were negative (2 NP, 1 BP), 2 with albuminuria (1 NP, 1 BP), 18 glomerular selective (11 NP, 7 BP), 2 glomerular non-selective (2 NP), 1 tubular (NP). In “CKD group”, 2 samples were negative (1 NP, 1 P), 8 with albuminuria (7 NP, 1 BP), 15 with glomerular selective pattern (12 NP, 1 BP, 2 P), 1 with glomerular non-selective (NP), 5 with tubular pattern (1 NP, 2 BP, 2 P) and 13 with mixed pattern (6 NP, 4 BP, 3 P).

The tubular bands were significantly more frequent (P=0.0006) in cats with CKD than in cats at risk (figure 68).

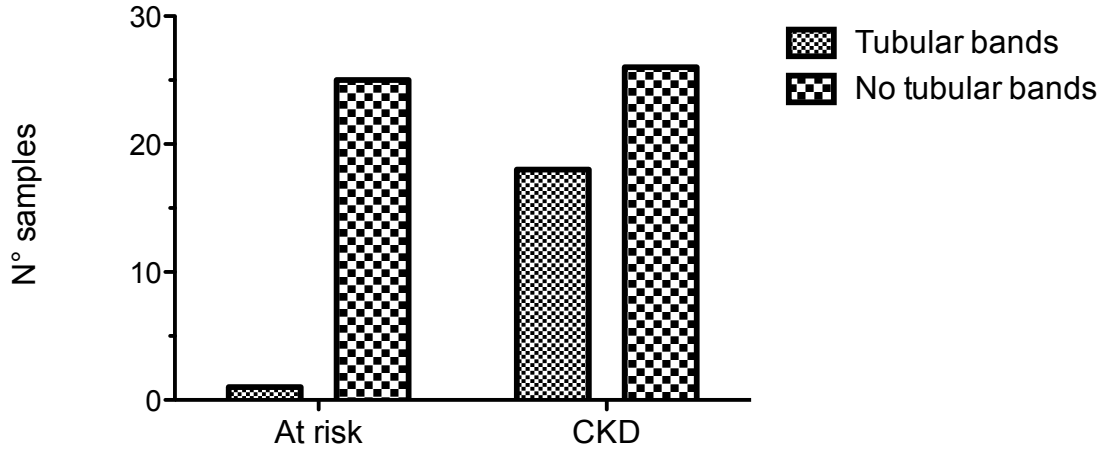


Figure 68 Distribution of data regarding number of samples with or without tubular bands in cats at risk and cats with CKD

Number of samples with tubular bands (tubular or mixed pattern) in the different groups dividend according to IRIS staging (including only CKD cats) is graphically displayed in figure 69. In figure 70, the distribution of UP, UPC ratio and USG in the same sub-groups is shown.

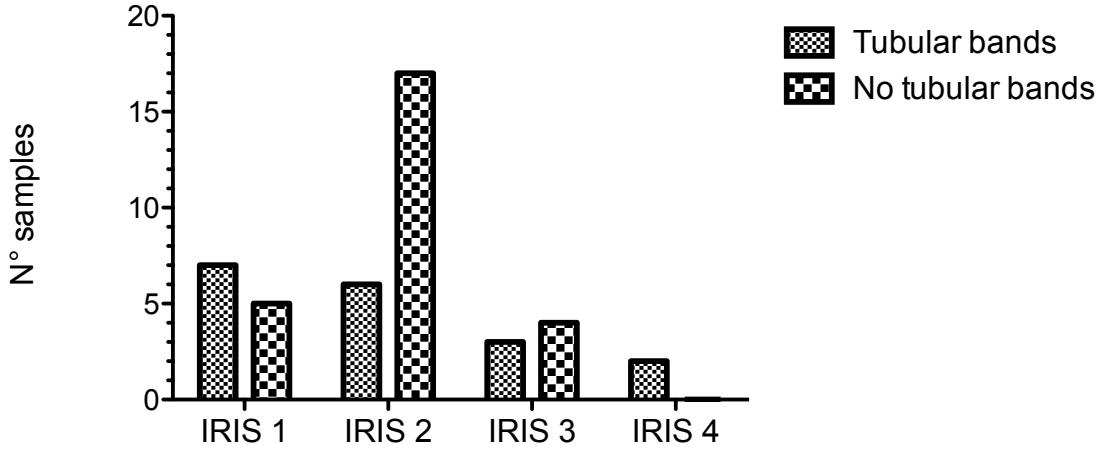


Figure 69 Distribution of data regarding number of samples with or without tubular bands in groups divided according to IRIS staging based on serum creatinine

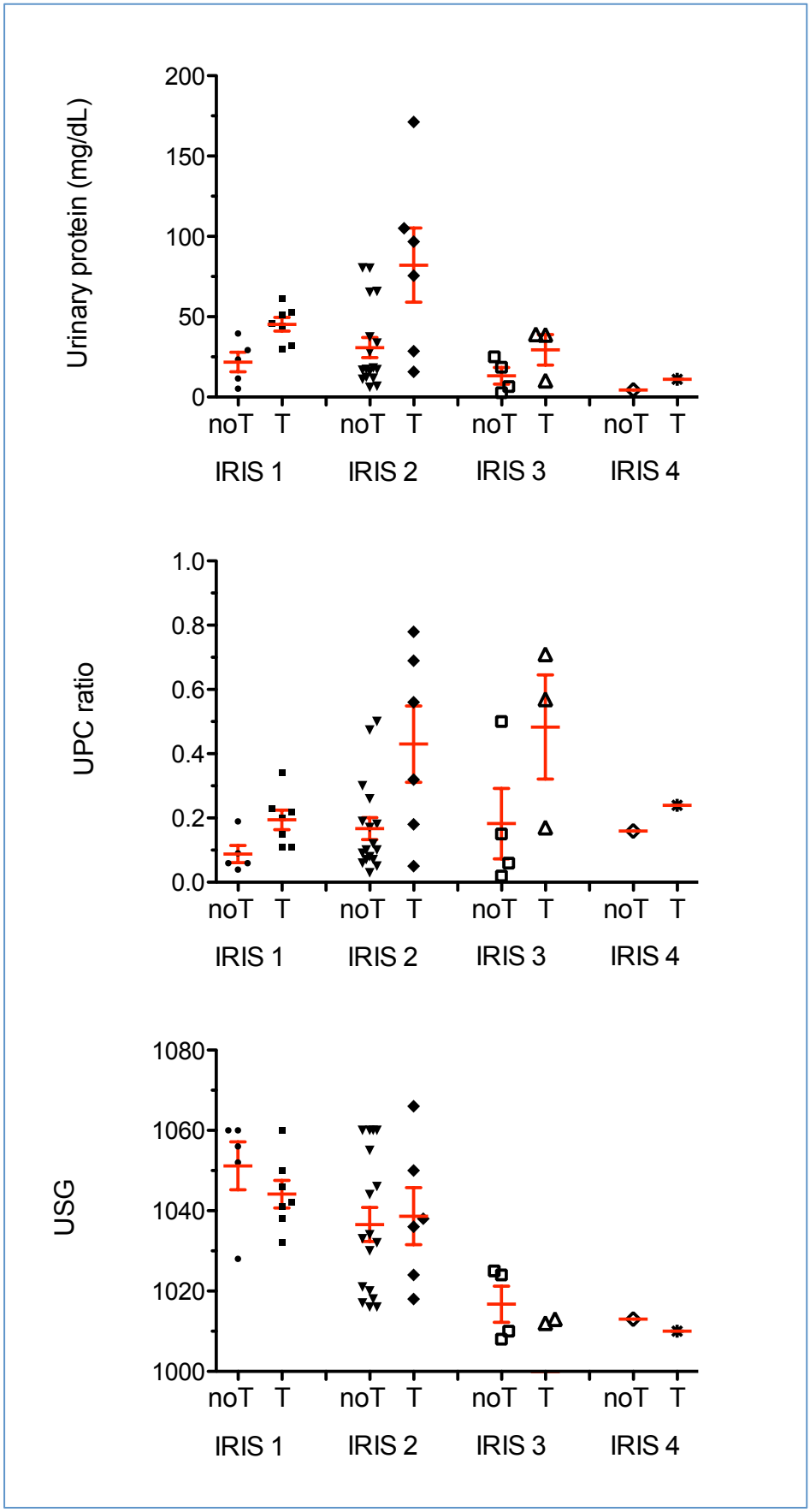


Figure 70 Distribution of urinary protein (top), UPC ratio (middle) and USG (bottom) groups divided according to IRIS staging and presence of tubular bands (noT: no tubular bands; T: presence of tubular bands

Considering all the samples, the tubular bands were also significantly more frequent (P=0.025) in proteinuric patients than in non-proteinuric (figure 71).

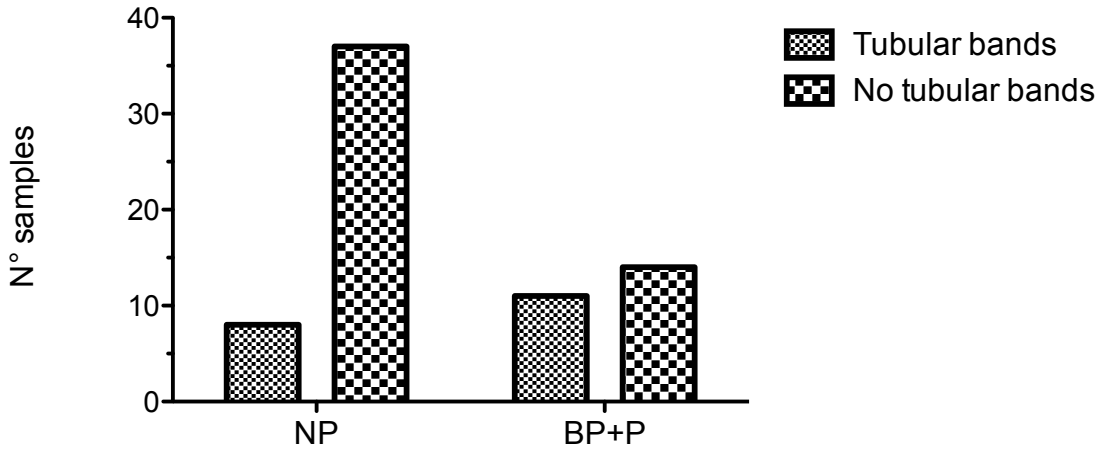


Figure 71 Distribution of data regarding number of samples with or without tubular bands in non-proteinuric (NP) group and in the group with UPC>0.2 (BP + P).

This different distribution was also significant (P= 0.0003) taking into account only CKD patients (Figure 72).

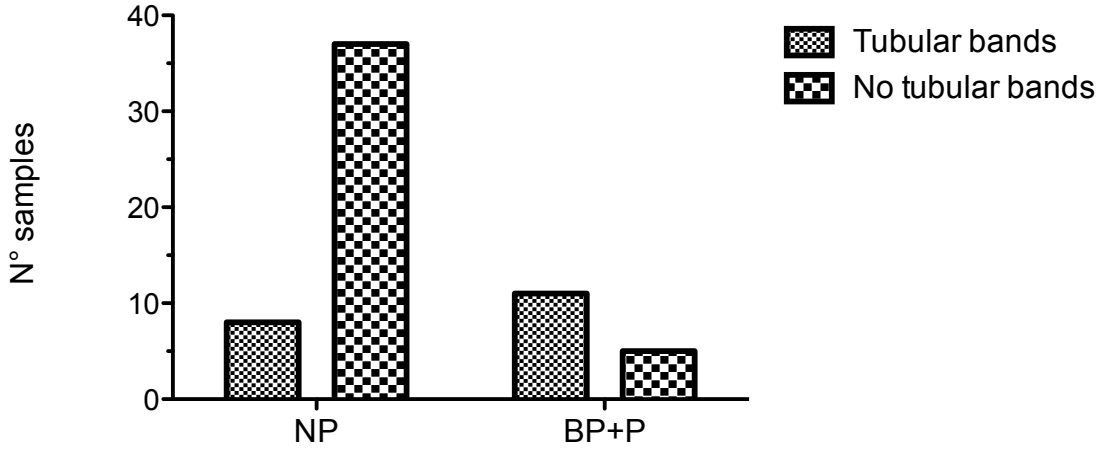


Figure 72 Distribution of data regarding number of samples with or without tubular bands in non-proteinuric (NP) group and in the group with UPC>0.2 (BP + P), including only CKD cats

Distribution of the tubular bands in the different groups divided according to IRIS stage for proteinuria (only CKD cats) is graphically displayed in figure 73.

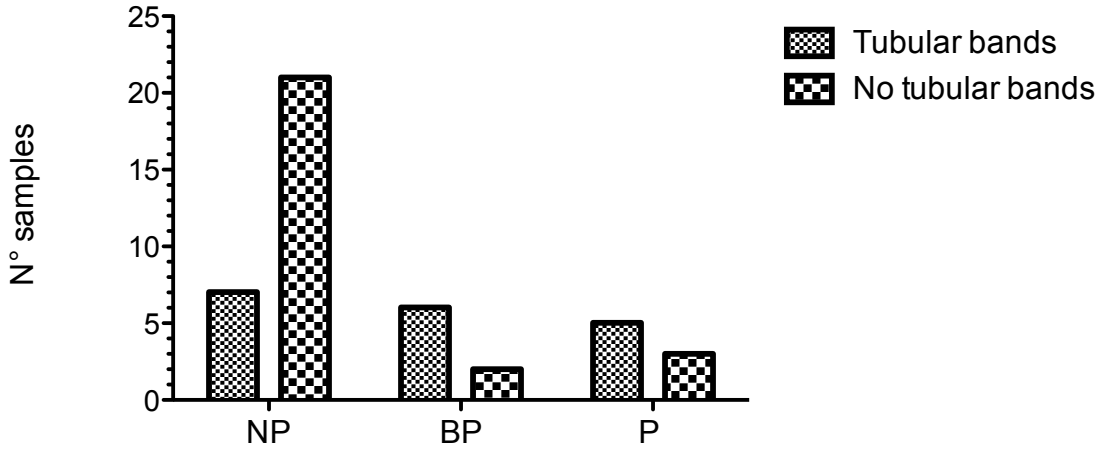


Figure 73 Distribution of data regarding number of samples with or without tubular bands in non-proteinuric (NP), borderline-proteinuric (BP) and proteinuric (P) group.

Discussion

Proteinuria was not so common in our population of cats with CKD. When present, it was mild in magnitude and never exceeded UPC =1. This is in accordance with a previous study in which 90% and 49% of cats with CKD had a UPC of <1.0 and <0.25, respectively (Syme et al., 2006).

The presence of proteins with MW equal or higher than albumin was frequent in our population. These were found even in the absence of proteinuria (i.e. UPC>0.2) or the absence of CKD, suggesting that the specificity is moderate in cats.

Since the majority of cats at risk having this pattern remained healthy for months after urine collection, the presence of these proteins and these patterns should be considered normal in cats, at least in the elderly cats at risk to develop CKD.

It is not possible to exclude early glomerular damage in our population but it is mild and uncommon in cats with CKD, especially at early stages (Brown e tal., 2016).

The presence of proteins with MW higher than albumin is already described in healthy cats (Ferlizza et al., 2015). The most abundant are cauxin (Miyazaki et al., 2003) and uromodulin (Ferlizza et al., 2015) and it is possible that these proteins are responsible of these findings in our population.

The tubular pattern, together to the mixed pattern, was significantly more frequent in CKD cats. It is also possible to note that tubular bands were identified at any stage of CKD in

our population and it could suggest that tubular damage occurs since the beginning of the disease in cats (Chakrabarti et al., 2013).

In addition, although few samples were included in IRIS stage 3 and 4, low MW proteins were relatively more frequent at late stages. Assuming that that the progression of CKD is strictly related to the worsening of the tubule-interstitial damage in cats (Brown et al., 2016), this latter results is in accordance to what found in in dogs with CKD, where the presence of low MW proteins (tubular bands) was significantly associated with the grade of tubule-interstitial damage (Zini et al., 2004)

Moreover, the higher frequency of tubular bands detected in proteinuric patients support the theory that proteinuria in nephropatic cats is mainly secondary to tubule-interstitial damage (Brown et al., 2016). As further confirmation, at any stage the UPC tended to be higher in samples with tubular bands compared to samples without tubular bands in the same IRIS stage.

Finally, it is interesting to note that the frequency of tubular damage in borderline-proteinuric and proteinuric cats is similar. This result suggests that BP in CKD cats need to receive more attention.

However, few samples are included in these groups and further statistical evaluations are needed to confirm this hypothesis.

General conclusions

This study had the aim to assess serum and urinary biomarkers usefulness in cats with CKD with or without proteinuria and hypertension.

Evaluation of big-ET1 in serum of cats did not show satisfying results, since very low concentration was found in both healthy cats and cats with CKD, but further evaluations are needed. Conversely, results in urinary samples were encouraging and, to our knowledge, this is the first study validating and detecting this biomarker in urine of cats with CKD. The evaluated ELISA method yielded satisfying validation results, supporting its introduction in this specie. The association between the urinary levels of Big-ET1 and the severity of CKD and proteinuria revealed that it could be a promising aid in nephropathic cats, especially at early stages. Moreover, it could shed light on the pathogenesis of tubulo-interstitial and glomerular damage in cats with CKD. Hence, it could reflect the inflammatory activity of the kidney as demonstrated in humans (Dhaun et al., 2009).

The enzymatic method for Hcy measurement, previously validated in dogs in our institution (Rossi et al., 2013), can be considered reliable also in cats according to the good precision and accuracy detected with the validation tests. From a diagnostic point of view, the results were however less encouraging in cats with hypertension and CKD when compared to other species, since no direct relationship with hypertension was found, suggesting that Hcy is not a valuable biomarker for hypertension in these patients. The progressive increase of Hcy concentration associated with the progressive increase in severity of CKD and the detection of high Hcy in some non-azotemic patients with CKD could add a new marker to those currently available for the identification and staging of the kidney disease of the cat, even though serum creatinine may provide superimposable information and may continue to be preferred to this new test.

Aldosterone was not associated with CKD and hypertension, although a tendency to increase in aldosterone in the most advanced stages of nephropathy was observed. These results were partially in accordance with previous studies performed on cats, where RAAS activation appeared to be not completely dependent on kidney injury (Steele et al., 2002; Jepson et al., 2014). The lack of a relationship with systemic blood pressure confirms that the pathogenesis of CKD-associated hypertension is multifactorial and is not caused solely by the activation of RAAS (Javadi et al., 2005). Therefore, Aldosterone cannot be considered an indicator of hypertension in cats affected with CKD and it is not useful in distinguishing real hypertension from white-coat hypertension. However, in the light of our

results and results of previous studies, aldosterone quantification can still be considered in patients with advanced CKD or in patients with non-responsive hypertension, given the possible high concentration in some of these patients.

The ELISA kit for A1MG measurement failed all the validation tests, therefore results were considered unacceptable. Accordingly, this commercially available ELISA could not be used, and further studies are needed in order to investigate the presence of A1MG in cats with CKD and tubular damage.

SDS-AGE showed the presence of bands corresponding to proteins with MW higher than albumin but lower than IgG in elderly healthy cats. Although an early glomerular damage could not be excluded in our population, this pattern appeared to be normal in this specie and has to be taken into account in the evaluation of proteinuria in cats. Tubular bands were common in patients with CKD at any stage confirming the predominant tubule-interstitial damage typical of this disease in cats. The presence of this pattern also at early stages (such as IRIS 1 and/or non-proteinuric and borderline proteinuric sub-stages) is promising and therefore SDS-AGE can be considered a valuable aid in the diagnostic approach to feline CKD. However, further studies including higher number of patients with early CKD and different urinary tract diseases are necessary to better evaluate the diagnostic utility of this test.

Scientific activities

International peer reviewed papers:

- **Giraldi M**, Paltrinieri S, Zatelli A. Evaluation of the analytical variability of protein pads on canine urine. *Veterinary Clinical Pathology*. IN PRESS
- **Giraldi M**, Rossi G, Paltrinieri S, Bertazzolo W, Scarpa P. Evaluation of the analytic variability of urine protein-to-creatinine ratio in cats. *Veterinary Clinical Pathology.* SUBMITTED
- Paltrinieri S, **Giraldi M**, Prolo A, Scarpa P, Beccati M, Graziani B, Bo S. Serum symmetric dimethylarginine (SDMA) and creatinine in Holy Birman cats compared with cats of other breeds. *Journal of feline medicine and surgery*. IN PRESS
- Hernandez A, Bilbrough GEA, DeNicola DB, Myrick C, Edwards S, Hammond JM, Myers AN, Heseltine J, Russell K, **Giraldi M**, Nabity MB. Comparison of the performance of the IDEXX SediVue Dx® with manual microscopy for detection of cells and crystals in urine sediments. *Journal of veterinary internal medicine*. SUBMITTED

Abstracts:

- **Giraldi M**, Rossi G, Scarpa P, Bertazzolo W, Paltrinieri S. Evaluation of proteinuria in cats: comparison between Coomassie Brilliant Blue and Pyrogallol red Molybdate. 25th ECVIM-CA / 17th ECVCP Congress – Lisbon (Portugal); 9th–12th September 2015

- **Giraldi M**, Paltrinieri S, Zatelli A. Evaluation of analytical variability of dipstick in identification of proteinuria. 26th ECVIM-CA Congress – Goteborg (Sweden); 8th–10th September 2016

- **Giraldi M**, Scarpa P, Paltrinieri S. Frequency of electrophoretic changes in urine of old cats with or without CKD. 18th ECVCP / ESVONC congress – Nantes (France); 20th–22nd October 2016

- **Giraldi M**, Rossetti P Vitiello T, Scarpa P. Reproducibility of urinary sediment examination: stain versus bright-field and phase-contrast. 27th ECVIM-CA congress – St. Julian's (Malta); 14th–16th September 2017

- Ruggerone B, Scarpa P, **Giraldi M**, Paltrinieri S. Reference intervals in Shetland sheepdogs: is primary hyperlipidemia a real features in this breed? 26th ECVIM-CA Congress – Goteborg (Sweden); 8th–10th September 2016

- Scarpa P; Palestrini C; Marelli SP; **Giraldi M**; Ghiringhelli M; Raja M; Fusi E. How Does the Nutritional Assessment of Dogs Vary in a Veterinary Staff? 26th ECVIM-CA Congress – Goteborg (Sweden); 8th–10th September 2016
- Borromeo V, Ocar O, Berrini A, Faverzani S, Rizzi R, **Giraldi M**, Scarpa P. Development and validation of a single blood sampling method of iohexol plasma clearance for the assessment of glomerular filtration rate (GFR) in dogs. 71th Convegno SISVET – Napoli (Italy); 28th June –1st July 2017
- Zambarbieri J; **Giraldi M**, Ruggerone B, Faverzani S; Scarpa P. Symmetric dimethylarginine (SDMA) and nephropathy in dog: diagnostic utility in clinical practice. 27th ECVIM-CA congress – St. Julian's (Malta); 14th–16th September 2017

Oral presentations:
- **Giraldi M**, Scarpa P. Case report. SIMIV congress – Cremona (Italy); March 7th-8th, 2015
- **Giraldi M**, Patrinieri S, Rossi G, Bertazzolo W, Scarpa P. Evaluation of proteinuria in cats: comparison between Coomassie Blue and Pyrogallol Red-Molybdate. Veterinary and Animal Science Days – Milan (Italy); 16-17th July 2015
- **Giraldi M**, Patrinieri S, Scarpa P. Evaluation of a commercial ELISA for measurement of feline urinary alpha1-microglobulin. Veterinary and Animal Science Days – Milan (Italy); 9-10th June 2016

Lecturer:
- Participation as speaker in 3 webinars for *Farmina pet foods*® lecturing about diagnosis and treatment of canine and feline chronic kidney diseases

Co-supervision of Degree thesis

- Stefano Negri: Rosso pirogallolo e blu di coomassie:due colorazioni a confronto per la determinazione del rapporto proteinuria/creatininuria (UPC) nel gatto**.** Thesis supervisor: Prof. Paola Scarpa; Thesis co-supervisor: Marco Giraldi. February 2016
- Paola Rossetti: Riproducibilitá dell'esame del sedimento urinario nel cane: confronto tra diversi metodi per la valutazione semiquantitativa di eritrociti e leucociti. Thesis supervisor: Prof. Paola Scarpa; Thesis co-supervisor: Marco Giraldi. July 2016

- Carola Curcio: Omocisteinemia nel gatto: validazione analitica di un metodo enzimatico e indagini preliminari sull'iperomocisteinemi in gatti nefropatici Thesis supervisor: Prof. Saverio Paltrinieri; Thesis co-supervisor: Marco Giraldi. October 2017
- Alessia Bellintani: Aldosterone nel gatto nefropatico: Osservazioni preliminari Thesis supervisor: Prof. Paola Scarpa; Thesis co-supervisor: Marco Giraldi. October 2017
- Camilla Piazza Thesis supervisor: Prof. Paola Scarpa; Thesis co-supervisor: Marco Giraldi. October 2017

Awards:

Best poster presentation at the 27th ECVIM-CA congress in the section clinical pathology (St. Julian's, Malta; 14th–16th September 2017).

Externship

I spent two months (June-July 2017) at the department of Veterinary Pathobiology at Texas A&M University (Texas, USA) under the supervision of Dr. M. Nabity (dipl. ACVP), where I was involved in a research project about the analytical validation of an automated analyser of canine and feline urine sediment. I took also part in weekly journal clubs of veterinary clinical pathology, seminaries and cytology-histology slide review rounds.

Other activities

During the three years I spent time in daily practice at the DIMEVET, under the supervision of Prof. Paola Scarpa, performing clinical consultations and laboratory works. In this period, I managed 186 primary clinical cases (both dogs and cats). I also had the opportunity to perform and interpret 636 haematological samples, 176 biochemical panels and 91 urine samples of feline and canine patients presented for clinical consultations at the DIMEVET, under the supervision of Prof. Saverio Paltrinieri. I also followed veterinary students which attended small animal internal medicine clinic and clinical pathology laboratory during my PhD period, tutoring them in their clinical and laboratory training.

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