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Physical Doctorate thesis in Veterinary Medicine:

"Early diagnostic and prognostic markers of chronic kidney disease (CKD) in canine and feline patients"

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A Zibi, Pluto, Piccolo e tutti i miei pazienti per i quali non sono riuscita a fare di più

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CHAPTER 1: SUMMARY

Chronic kidney disease (CKD) is a clinical syndrome with a high prevalence both in human and veterinary medicine. Being able to formulate an early diagnosis of CKD can allow veterinarians to introduce a dietary and medical therapy, which can dramatically reduce progression towards end stage renal disease (ESRD)[1]. At the same time, strong evidences of a deep relationship between heart and kidney in the progression of CKD in humans, have led to a greater attention towards potential markers of prognosis and negative outcome of the disease, even in small animal population. The present research project has been organized in two sections[2,3].

FIRST SECTION

The aim of the first part of the study has been to validate a glomerular filtration rate (GFR) method, at a low number of plasma samples, through the plasma clearance of iohexol in both CKD and clinically healthy cats.

MATERIALS AND METHODS - After the owners' informed consent, 53 clinically healthy and 14 CKD cats have been submitted to a first blood sample (0) and to an eight-hour clearance study. Iohexol (Omipaque® 300 mgI/ml) has been intravenous injected at the dose of 64.7 mg/kg body weight. Heparinised blood samples have been taken at 5 and 30 minutes and 1, 2, 4, 6 and 8 hours from the completion of iohexol injection. Plasma has been obtained and each sample has been stored at -20 C° till extraction process and HPLC analysis. Pharmacokinetic analysis has been performed through the software Easy Fit® for Macintosh (Istituto Mario Negri, Milano, Italia) and, for each subject of HC and CKD group, plasma concentration of iohexol/time curves have been analyzed through a non-compartimental kinetic model. Then, a pharmacokinetic analysis has been carried on after the application of simplified models (Model A, Model B, Model C and Model D) with a lower number of blood samples. Statistical analysis has been performed by using the software GraphPad Prism® 4 for Macintosh, USA.

RESULTS – t-test analysis ($p<0.05$) between GFR of CH and CKD patients has shown a significant difference between the two groups of subjects, not only for reference method

(p=0.003), but also for Model A (p=0.0005), Model B (p=0.01), Model C (p=0.001) and Model D (p=0.004). Pearson correlation analysis ($p<0.05$) between each simplified model and reference method has shown a positive linear correlation with vey high values of Pearson r and R^2.

CONCLUSIONS - The present study has validated a safe, simple and accurate three-sample HPLC method (5' – 30' – 1 hour) for the determination of GFR through the plasma clearance of iohexol in feline patients. This model represents an attractive and cheap alternative to cumbersome plasma clearance methods, with a dramatic applicatory potential in different clinical settings.

SECOND SECTION

The aim of the second part of the study has been to assess serum ionized calcium, total calcium, calcium corrected for albumin (cCaAlb), calcium corrected for total proteins (cCaPt), Ca x P product (Ca x P), cardiac troponin I (cTnI), C-reactive proteine (CRP) and α-tochopherol in CH and CKD canine patients at different stages of the disease.

MATERIALS AND METHODS – serum ionized calcium, total calcium, cCaAlb and cCaPt have been determined in 301 CKD and 125 CH patients, while Ca x P, cTnI, CRP and α-tochopherol have been assessed in 13 IRIS 1, 7 IRIS 2, 13 IRIS 3 and 11 IRIS 4 subjects. Ionized calcium has been determined through a selective ion method (STAT PROFILE® pHOx Plus, GEPA, Milano, Italy), cTnI through an immunometric method (IMMUNOLITE 2000® Immunoassay System), CRP through an immunometric method (RANDOX immunoturbidimetric kit® for CRP, Vet Med Lab, IDEXX, Germany) and α-tochopherol through HPLC (Chromosystems-Diagnostic Kit HPLC & LC/MS®, Munchen, Germany). Statistical analysis has been performed by using the software GraphPad Prism® 4 for Macintosh, USA.

RESULTS – One-way ANOVA has reported a significant difference ($p<0.0001$) in ionized calcium concentration among CH, IRIS 1, IRIS 2, IRIS 3 and IRIS 4 and χ^2 analysis has shown a significant difference ($p<0.0001$) in the number of patients with hyper, hypo and normocalcemia according to the progression of the disease. One-way ANOVA among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients has reported a significant difference in the mean value of Ca x P ($p<0.0001$). No significant correlation has been found between Ca x

P and plasma creatinine in any of IRIS classes. The number of patients with Ca x P above 70 mg/dl has been reported to increase significantly ($p<0.0001$) with the severity of CKD, as well as the number of dead patients ($p<0.0008$). Finally, Kaplan-Meier survival curve has shown a significantly higher percentage of survival ($p<0.0002$) of CKD patients with Ca x P below 70, compared to patients with Ca x P above 70. One-way ANOVA among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients has reported a significant difference in the mean value of cTnI ($p<0.02$). No significant correlation has been found between cTnI and plasma creatinine in any of IRIS classes. The number of patients with cTnI above 0.20 ng/ml has been reported to increase significantly ($p<0.0001$) with the severity of CKD, as well as the number of dead patients ($p<0.02$). Finally, Kaplan-Meier survival curve has shown a significantly higher percentage of survival ($p<0.0002$) of CKD patients with cTnI below 0.20 ng/ml, compared to patients with cTnI above 0.20 ng/ml. One-way ANOVA among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients has reported a significant difference in the mean value of CRP ($p<0.0001$). No significant correlation has been found between CRP and plasma creatinine in any of IRIS classes. The number of patients with CRP above 9.7 mg/l has been reported to increase significantly ($p<0.0001$) with the severity of CKD, as well as the number of dead patients ($p<0.0009$). Finally, Kaplan-Meier survival curve has shown a significantly higher percentage of survival ($p<0.001$) of CKD patients with CRP below 9.7 mg/l, compared to patients with CRP above 9.7 mg/l. One-way ANOVA among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients has reported a significant difference in the mean value of α-tochopherol ($p<0.0002$). A significant correlation ($p=0.00$) has been found between α-tochopherol and plasma creatinine in IRIS 2. The number of patients with α-tochopherol below 21.6 ppm has been reported to increase significantly ($p<0.0001$) with the severity of CKD. No significant difference in the number of survived and dead patients has been found between subjects with α-tochopherol below and above 21.6 ppm. Finally, Kaplan-Meier survival curve has shown no significant difference.

CONCLUSIONS – The present study has demonstrated a significant increase in Ca x P, cTnI and CRP serum concentration according to the progression of CKD. Ca x P, cTnI and CRP have shown a prognostic, not IRIS stage-dependent, value and a significant correlation towards mortality. In CKD dogs, as well as in humans, alterations of calcium-phosphate metabolism, cardiovascular injury and inflammation seemed to play a significant role in the

progression and negative outcome of CKD. No correlation has been reported between mortality and α-tochopherol, although a significant serum reduction with the progression of CKD has been shown.

CHAPTER 2: PREFACE AND AIM OF THE STUDY

Chronic kidney disease (CKD) is a complex clinical syndrome, with an even increasing prevalence in both human and veterinary medicine[1,2]. According to human guidelines, CKD has been defined as a pathological condition characterized by a persistent renal injury or a glomerular filtration rate (GFR) below 60 ml/min/1.73m^2, lasting for four to more months, with or without evidence of kidney damage, irrespective of the cause. CKD is becoming a major public health problem worldwide, which affects about 7.2% of people below 30 years old and a 23.4% to 35.8% of people above 64 years old[3]. In human patients glomerulonephritis has been the leading cause of CKD since several decades ago, while, at present, infections have been showing an even lower importance in the pathogenesis of the disease, at least in the western world. On the contrary, the role of hypertension and diabetes in causing CKD has been increasing significantly. In addition, CKD presents wide interrelationships with other pathological diseases. Recent reports have underlined that CKD is an independent risk factor of cardiovascular disease and the American Heart Association stated that persons with CKD should be considered as the highest risk group for developing subsequent cardiovascular diseases[3,5]. The presence of a cardio-renal syndrome, characterized by a deep interconnection between kidney and heart injury, has been firstly postulated, then widely demonstrated. Oxidative stress, inflammation and alterations in calcium-phosphate metabolism have been reported both as risk factors and progressive elements of the cardio-renal syndrome[6].

In veterinary medicine, as well, CKD represents an even more frequent reason of consultation. Its prevalence in feline and canine population has been estimated about 1-3% and 0.5-1.5% respectively. When geriatric patients are considered, the prevalence of CKD can reach 10% in dogs and 35% in cats. Moreover, recent reports have showed that, on a ten-year period of clinical activity, the 24% of canine patients, presented for consultation, were affected by CKD[7]. Since 2006, the International Renal Interest Society (IRIS) has introduced a standardized classification of CKD, based on serum creatinine. According to IRIS classification, both canine and feline patients can be classified in four stages of severity[8]. Previously reported epidemiologic studies have taken into consideration only the stages of

CKD with plasma creatinine above the reference range. So, no data are available about the prevalence of early phases. IRIS stage 1 is, in fact, a totally asymptomatic condition, which can be identified only by using GFR. Although in veterinary medicine, as well as in human medicine, haemodialysis and renal transplantation are becoming an interest reality all over the world, limitations due to high costs and low availability of specialized centers have focused the attention on early diagnosis. The establishment of an even earlier diagnosis of CKD can allow veterinarians to introduce a dietary and medical therapy, which can reduce the progression of patients towards end stage renal disease (ESRD). At the same time, strong evidences of a deep relationship between heart and kidney in the progression of CKD in humans, have led to an even more attention towards potential markers of prognosis and negative outcome of the disease, even in small animal population.

In the first section of the present work, the aim of the study has been to identify a simplified GFR evaluation method by the plasma clearance of iohexol, based on a low number of plasma samples, in both clinically healthy and chronic kidney disease cats.
In the second section of the research project, the aim of the study has been to evaluate the serum concentration of Ca x P product, cardiac troponin I, C-reactive protein and α-tochopherol in canine patients at different stages of chronic kidney disease and to test their prognostic value.

CHAPTER 3: CHRONIC KIDNEY DISEASE (CKD): IMPORTANCE OF AN EARLY DIAGNOSIS

Chronic kidney disease (CKD) refers to a pathological loss of functional renal tissue, characterized by a progressive and prolonged morbid event. Most forms of CKD are not reversible and, once acquired, they generally proceed to renal failure. The prevalence of CKD in the small animal population is estimated about 1-3% of cats and 0.5-1.5% of dogs and its incidence strongly increases with advancing age. In geriatric population the prevalence of CKD can reach a percentage of 10% of dogs and 35% of cats[1]. The vascular and tubular components of nephrons are strictly interdependent and CKD represents the end-point of an irreversible glomerular or tubular damage. Although the histopathological findings are often not process-specific, primary glomerular disorders have been identified as a frequent cause of CKD in dogs[2].

The major role of the kidneys is to maintain the normal volume and composition of the extracellular fluid by filtering the blood through the glomeruli and adjusting the concentration of the substances into the filtrate. At the same time kidneys control the local glomerular haemodynamics, sodium reabsorption and systemic blood pressure through the activity of the renin-angiotensin-aldosterone system (RAAS). Moreover, the production of erythropoietin (EPO) and the activation of calcitriol have to be enrolled among the renal functions. Kidneys are so involved in the stimulating activity of production of erythrocytes and in the intestinal absorption of calcium. As the kidney shows multiple functions, there is not a single test able to assess all of them simultaneously. Whereas many tests are currently available for the evaluation of the different renal functions, the clinician has to choose the most appropriate one, according to the purpose. In choosing the right test to use, it is essential to take into consideration the difference between kidney lesions and renal impairment. As a damaged kidney may have a normal function and CKD may show no visible lesions, it is possible that visible kidney injuries exist in the absence of renal dysfunction and vice versa. Patients affected by pre-renal acute renal failure (such as with a severe dehydration) can show a significant reduction of the GFR even though no lesions can be found in the renal tissue. Some tests are more useful than others depending on the course of the disease. Ultrasound and

renal biopsy can be more helpful in the diagnosis of ARF, while functional tests can play a fundamental role as diagnostic indexes of CKD[1]. Though the multiple renal functions cannot be assessed simultaneously, GFR is universally recognized as the most accurate marker of the overall renal function. GFR is a precise and direct test of glomerular filtration, which can detect a decreased kidney function before the onset of a CKD status[3]. GFR is directly related to the functional renal mass of the patient and allows a quantitative evaluation of the overall kidney function both in healthy and in nephropatic subjects. Canine and feline kidneys are constituted by a big number of nephrons, which work together side by side. It is possible that a loss of a certain number of nephrons occurs without any clinical evidence of renal failure. Kidneys show a great ability to compensate for a significant lack of renal mass by increasing the activity of the survived nephrons. More than 50% of the total renal tissue has to be destroyed before lacking the urinary concentration ability and more than 70% before developing clinical symptoms of CKD[4,5].

Glomerular filtration rate (GFR): gold standard test of overall renal function

For a long period of time GFR has been estimated through the clearance of blood urea and creatinine. Although these parameters provide just a crude index of GFR they are still largely use for the diagnosis of CKD by the majority of veterinarians (6). Urea and creatinine show a very low sensibility in predicting GFR. Correlation studies performed between blood urea and GFR and between blood creatinine and GFR reported a significant dispersion, showing a poor ability to estimate GFR (7). The compensatory hyper-filtration of survived nephrons is responsible for a late haematic increase of creatinine and urea concentration. The blood value of creatinine starts to rise when more than 65-70% of the total functional renal mass has been definitely lost (6-8-9). GFR can be calculated through the evaluation of plasma or urinary clearance of a substance. An ideal marker of GFR should be freely filtered by the glomerulus (not protein bound) and not affected by tubular reabsorbing process or secretion or be metabolized elsewhere in the body. In addition the marker must not be toxic for the patient. At present, in veterinary medicine, GFR can be assessed through different methods, which present both advantages and disadvantages. In canine patients the urinary clearance of inulin

is considered the gold standard method for the GFR evaluation. Inulin does not bound to plasma proteins and shows no significant metabolism. This compound is freely filtrated through the glomerulus and not reabsorbed or secreted by the tubulus. Inulin shows a negligible toxicity and several studies reported no side effects in treated animals (9-10-11). Nevertheless, the urinary clearance of inulin is a cumbersome method, which requires an extremely accurate urine sampling. Moreover inulin is an expensive compound, not easily available in Europe, which needs special equipments to be analyzed. The urinary clearance of inulin, such as other urinary methods (endogenous and exogenous creatinine), shows several disadvantages due to urine collection. These methods require urine collection for a lengthy period (i.e. 24 hours) to minimize errors in the collection, thus necessitating indwelling catheters, repeated catheterization or metabolic cages. The use of urinary catheterization and metabolic cages can cause urinary infections and be extremely stressful for patients (10-6). Endogenous creatinine is freely filtered by the renal glomerulus and it derives from a constant breakdown of muscle creatine phosphate. Although no protein binding is present, endogenous creatinine clearance can be influenced by several factors, such as gender, age, season, biological rhythm, hydration, physical exercise and site of blood sample. Furthermore, plasma and serum contain other compounds, which are inappropriately measured as creatinine, when Jaffe reaction method is used. In addition, male dogs have shown a small degree of renal tubular secretion of creatinine. In order to reduce problems connected with endogenous creatinine clearance, methods based on exogenous creatinine clearance have been developed. Initially these methods have been performed through constant rate infusion or subcutaneous techniques, then a single intravenous bolus way has been proposed[20]. Constant infusion method has reported to underestimate GFR by as much as 18.8% in feline patients[21]. Single-bolus plasma clearance of exogenous creatinine has been accepted as an accurate and simple method to determine GFR[22,23]. Anyway, it has to be underlined that, in order to obtain accurate measurements of GFR, this method should extend over a ten-hour period with a minimum of three to five samples[24]. Radioactive GFR markers, such as ^{51}Cr-ethylenediaminetetraacetic acid (EDTA) and ^{99m}Tc-diethylenetriaminepentaacetic acid (DTPA), fulfil all characteristics of ideal markers and can allow a contemporary renal scintigraphy, in order to assess overall and single renal function. Unfortunately these methods show multiple disadvantages, including low availability of laboratories, appropriate facilities

to conduct nuclear medicine-based tests and high intra-dog variability[20]. Radioactive markers, in fact, need gamma camera to be detected, so operator inexperience may cause significant lack in accuracy of GFR measurement[25]. Finally, GFR can be assessed through plasma clearance of iohexol. Iohexol is a low osmolality, non-ionic, iodinated contrast medium, which can be determined by using different techniques. High plasma stability of iodinated compounds allows them to be frozen for extended times. Although, different detecting techniques have shown significant differences in GFR result, according to quantification of endo- or exo-iohexol, high performance liquid chromatography (HPLC) system has demonstrated the more accurate and high reproducibility method[26,27]. Moreover, iohexol has been generally considered very safe even in severely nephropatic subjects [28,29]. Plasma clearance of iohexol has been considered a valid method of GFR determination in both dogs and cats. At present, a two plasma sample and two-hour method in dogs and a two-hour plasma sample and three-hour method in cats, are considered the best compromise between accuracy and easy applicability of the method[30]. Very recently, an automated colorimetric gadolinium assay for GFR evaluation has been investigated in canine and feline patients[31]. Gadolinium contrast media are a high osmolality compounds, which have been widely used as contrast agents in magnetic resonance imaging (MRI) in both humans and companion animals. Anyway, their use in CKD patients is contraindicated. High osmolality contrast media gadopentetate have caused severe renal function depression, when injected into renal arteries. Radiographic contrast media, in fact, can cause an acute renal failure syndrome. The most important nephrotoxic mechanisms of such compounds have been identified in hyperosmolality and osmotic diuresis. High osmolality solutions, which are not able to cross cell membranes, can be responsible for a renal damage, which ranges from a transient depression of renal blood flow and GFR to extensive macro and microscopic changes with cessation of renal function. This action depends on the dose and the degree of hyperosmolality of the contrast media. Osmotic diuresis, instead, can be generated by contrast media molecules, once they have reached tubular lumen. As high osmolality contrast media compounds are not reabsorbed by renal tubules, they may act as osmotic diuretics. Osmotic load may be toxic for kidneys by activating the tubulo-glomerular feedback mechanism, which activates vasoconstrictive agents, by increasing oxygen demand for active reabsorption of the high tubular sodium load, by increasing intratubular pressure, with elevation of

interstitial pressure and secondary vascular compression beneath the rigid renal capsule[32]. At present, low osmolality contrast media are strongly preferred to high osmolality ones, due to significantly lower prevalence of side effects[32].

Urinary clearance methods requires the determination of both plasma and urinary concentration of the used marker. As a consequence, all urine must be collected over the entire clearance study. An incomplete or inaccurate urine collection leads to an underestimation of GFR. 24-hour urine collection and consequent required techniques, make these methods very difficult to be performed in companion animals. For this reason, plasma clearance methods have shown more utilized in veterinary medicine. In a plasma clearance study, GFR is obtained by determining the reduction in plasma concentration of a marker over time. Plasma clearance (CL_{plasma}) of a marker is calculated by the formula $CL_{plasma}= D/AUC$, where D corresponds to the dose of the injected marker and AUC to the area under the plasma concentration versus time curve. Plasma disappearance curve is determined by multiple plasma samples. The resultant total area under the plasma concentration versus time curve is affected by the kind of pharmacokinetic model used. One-compartment model produces a straight line plasma disappearance curve, which needs a limited number of samples to be determined, as the line between any two points is assumed to be constant. Anyway an immediate distribution of a marker through the body does not occur in reality, so this model may generate errors[33]. In two-compartment model plasma tracer is supposed to move initially from vasculature to body tissues, than to be eliminated by the body. Resultant plasma concentration versus time curve is constituted by an initial steep segment (corresponding to the initial tissue distribution of the marker), followed by a less steep portion (corresponding to the elimination phase). Two-compartment model is highly accurate, because it takes into consideration both the distribution and the elimination phase. Anyway, the resolution of the curve into two segments is not possible in patients with extracellular fluid volume is abnormal[34]. Multi-compartment model is similar to two-compartment model, with the exception that, for each conceptualized compartment, an equal number of straight lines is required. Finally, non-compartment model calculates the area under the curve, by adding the area of each trapezoid defined by the curve itself. In this model the terminal portion of the curve (from the last plasma sample to the time of theoretical zero plasma concentration) is estimated.

GFR can be expressed to body weight, body surface area or extra-cellular fluid volume. In dogs GFR is more related to metabolic rate than to body weight, so it is advisable to express GFR as ml/min/m^2. Dogs have showed variable results depending on the utilized method of standardization, while most studies in cats have produced acceptable results for normalization with any method, probably due to their smaller range of body sizes[20].

CHAPTER 4: MATERIALS AND METHODS

Treatment of animals

The research project involved a total of 53 privately owned cats of different breed, gender, age and weight, presented to the Veterinary Teaching Hospital "Mario Modenato" between December 2008 and December 2010. 39 cats, presented for the annual general check up, resulted clinically healthy after clinical evaluation and showed no signs of polyuria/polydispsia (PU/PD). 14 cats were referred for nephrological consultation due to clinical signs of PU/PD, weight loss and plasma levels of creatinine > 1.6 mg/dl. After the owner's informed consent, all cats were submitted to clinical evaluation and blood sampling (5 ml) for the evaluation of WBC, RBC, Hct, Hg, plasma urea and creatinine, serum total and ionized calcium, phosphate, albumin and total proteins. According to the plasma value of creatinine (> 1.6 mg/dl) and the presence of PU/PD, patients were divided in clinically healthy (HC) and chronic kidney disease cats (CKD). 53 patients belonged to the HC group, while 14 cats to the CKD group. Cats were submitted to a twelve-hour fasting period in order to perform the routine haematological and biochemical panel. Each cat was accurately weighted and provided with two intravenous catheters at the cephalic veins. A first blood sample (0) was taken from one the catheters before injecting the contrast medium and blood (0.5 ml) was put into a heparinised tube. The syringe containing iohexol was weighted before collecting the contrast medium (empty weight), full of iohexol (pre inoculation weight) and after the injection (post inoculation weight), in order to determine the exact injected dose. For each patient an identification report was filled in. Iohexol (Omipaque® 300 mgI/ml) was slowly administered through the intravenous catheter at the dosage of 64.7 mg/kg in 60 seconds. The completion of the injection represented time 0 (t0). Each patient was submitted to seven blood samples (0.5 ml) at 5 and 30 minutes and 1, 2, 4, 6 and 8 hours from t0 and blood was collected in heparinised tubes. Samples were taken from the intravenous catheter opposite to the one used for iohexol inoculation.

Preparation of samples

Blood samples were centrifuged at 3000 rpm for 10 minutes in order to obtain plasma. Each plasma sample was classified and stored at -20°C till HPLC analysis. Samples were submitted to extraction. 50 µl of plasma were added to 50 µl of iopentol water solution (50 µg/ml) and, after a 60 second vortex time, samples were extracted through 100 µl of dichloromethane (CH_2Cl_2) and 150 µl of distilled water. Solution was vortex for 60''and centrifuged at 3000 rpm for 10 minutes. Supernatant was centrifuged and injected in HPLC.

Pharmacokinetic analysis

Results were analyzed through the software Easy Fit® for Macintosh (Istituto Mario Negri, Milano, Italia). For each subject of the HC group and the CKD group plasma concentration of iohexol/time curves were analyzed through a non-compartimental kinetic model. Then pharmacokinetic analysis was performed for each subject after the application of simplified models (Model A, Model B, Model C and Model D) with a lower number of blood samples. Model A was based on four samples at 5', 30', 1 and 4 hours, Model B was based on four samples at 5', 30', 1 and 2 hours, Model C was based on four samples at 5', 1, 2 and 4 hours and Model D was based on three samples at 5', 30' and 1 hour. Plasma concentration/time curves were analyzed by using a non-compartimental kinetic model.

Materials

Iohexol was used as intravenous water solution (Omnipaque® 300 mgI/ml-GE HEALTHCARE, GE Medical System, Milano, Italia). All solvents were suitable for a HPLC system (Baker Analyzed® Reagent, J.T. Baker, Deventer, Holland). Analytical water, used for reaching the right concentration of HPLC mobile phase, was filtered by using cellulose vinegar silicone proofed filters (PS Whatman®, Millipore Corporation, Maid Stone, England). All other reagents were bought from common commercial sources.

Tools

HPLC system included a binary gradient pump SpectraSYSTEM® P2000 Thermo Finnigan, a UV-VIS detector SpectraSYSTEM® 3000 Thermo Finnigan and an automatic sampler SpectraSYSTEM® AS3000 Thermo Finnigan (Waltham, MA, USA). The chromatographic column (Waters SunFire® 250 x 4,60 mm), with a granulometry of 5 μm, and the pre-column (Waters Guard-Pack™, Waters, Milford, MA, USA) were packed through a C18 alkylic group-derived silica gel. An injection volume of 100 μl was used.

Standard and stock solutions

Standard solutions of iohexol were prepared from the commercial water solution Omnipaque® 300 mgI/ml (64.7 mg/ml of iohexol), by which a 2 mg/ml mother solution was obtained. Standard solutions were obtained from mother solution through different dilutions (5, 10, 25, 50, 100 and 200 μg/ml) and calibration line was determined by injecting 100 μl in the HPLC system. Standard solutions of iopentol were prepared from the commercial water solution Imagopaque® 300mgI/ml (658 mg/ml of iopentol), by which a 2 mg/ml mother solution was obtained. Standard solutions were obtained from mother solution through different dilutions (5, 10, 15, 50, 100 and 200 μg/ml). Iohexol and iopentol solutions were stored sheltered from the light at 4°C.

Chromatographic conditions

Mobile phase was made by water at a pH of 2.6 and acetonitrile in a 94/6 % v/v ratio. The water pH of 2.6 was reached by adding H_3PO_4 at 85%. A 1.3 ml/min flow was used. Mobile phase was degassed through a nitrogen flow. Every time that mobile phase had to be replaced with a new one, chromatographic equilibrium was restored before performing HPLC analysis.

Calibration curves

Calibration lines of iohexol and iopentol were obtained through injection of the standard solutions, according to chromatographic conditions. Each solution was injected three times and the equation of the calibration curve was determined through the software Graph Pad Prism® (Graph Pad Software Inc., IL, USA), by using the mean value of three measurements.

Area under chromatographic peaks was measured through the software ChromQuest 4.2 (Thermo Finnigan, Waltham, MA, USA) and used as a quantitative parameter.

Statistical analysis

For both HC group and CKD group the average value of GFR (ml/min/kg) obtained by the application of the reference seven-sample method (reference method) and simplified models (Model A, Model B, Model C and Model D) was calculated. A t-test analysis between the GFR values of HC and CKD group was performed for GFR determined through the reference method and each of the four simplified models. For each group of patients (HC and CKD) Pearson correlation analysis ($p<0.05$) was carried out among reference GFR and the corresponding GFR value assessed through Model A, Model B, Model C and Model D. Similarly, a Bland-Altman analysis was performed among reference GFR and the corresponding GFR value determined through Model A, Model B, Model C and Model D in both HC and CKD patients. Statistical analysis was performed by using the software GraphPad Prism® 4 for Macintosh, USA.

CHAPTER 5: RESULTS

t-test analysis

HC group was composed by 39 cats. Plasma creatinine ranged from a minimum of 0.80 mg/dl to a maximum of 1.58 mg/dl (mean: 1.24 mg/dl). Reference GFR ranged from a minimum of 1.64 ml/min/kg to a maximum of 8.58 ml/min/kg, with a mean value of 3.25 ml/min/kg ± SE 0.27. Model A GFR ranged from a minimum of 1.52 ml/min/kg to a maximum of 6.80 ml/min/kg, with a mean value of 3.32 ml/min/kg ± SE 0.47. Model B GFR ranged from a minimum of 1.64 ml/min/kg to a maximum of 6.62 ml/min/kg, with a mean value of 3.28 ml/min/kg ± SE 0.38. Model C GFR ranged from a minimum of 1.40 ml/min/kg to a maximum of 6.87 ml/min/kg, with a mean value of 3.08 ml/min/kg ± SE 0.41. Model D GFR ranged from a minimum of 1.51 ml/min/kg to a maximum of 7.24 ml/min/kg, with a mean value of 3.43 ml/min/kg ± SE 0.39.

CKD group was composed by 14 cats with clinical signs of PU/PD and plasma creatinine above the reference range (1.6 mg/dl). Plasma creatinine ranged from a minimum of 1.87 mg/dl to a maximum of 3.00 mg/dl (mean: 2.54 mg/dl). Reference GFR ranged from a minimum of 1.45 ml/min/kg to a maximum of 3.81 ml/min/kg, with a mean value of 2.30 ml/min/kg ± SE 0.19. Model A GFR ranged from a minimum of 1.25 ml/min/kg to a maximum of 2.99 ml/min/kg, with a mean value of 2.11 ml/min/kg ± SE 0.17. Model B GFR ranged from a minimum of 1.49 ml/min/kg to a maximum of 4.17 ml/min/kg, with a mean value of 2.57 ml/min/kg ± SE 0.23. Model C GFR ranged from a minimum of 1.35 ml/min/kg to a maximum of 3.66 ml/min/kg, with a mean value of 2.28 ml/min/kg ± SE 0.19. Model D GFR ranged from a minimum of 1.36 ml/min/kg to a maximum of 4.24 ml/min/kg, with a mean value of 2.45 ml/min/kg ± SE 0.26.

	Plasma Cr *(mean)*	**Reference** *(GFR±SE)*	**Model A** *(GFR±SE)*	**Model B** *(GFR±SE)*	**Model C** *(GFR±SE)*	**Model D** *(GFR±SE)*
HC	1.24	3.25 ± 0.27	3.32 ± 0.47	3.28 ± 0.38	3.08 ± 0.41	3.43± 0.39
CKD	2.54	2.30 ± 0.19	2.11 ± 0.17	2.57 ± 0.23	2.28 ± 0.19	2.45± 0.26

Table 1: Mean values (ml/min/kg) of GFR and plasma creatinine (mg/dl) in HC group and CKD group. GFR is reported as mean value for reference method and for each simplified model (Model A, Model B, Model C and Model D). SE: standard error.

The t-test analysis ($p<0.05$) among GFR values of HC group and CKD group, according to reference method, showed a significant difference ($p=0.003$) between the two groups of subjects. Similarly, t-test analysis between GFR of HC group and CKD group assessed through simplified models, reported a significant difference. The t-test analysis ($p<0.05$) among GFR values of HC group and CKD group, according to Model A, showed a significant difference ($p=0.0005$) between the two groups of subjects. The t-test analysis ($p<0.05$) among GFR values of HC group and CKD group, according to Model B, showed a significant difference ($p=0.01$) between the two groups of subjects. The t-test analysis ($p<0.05$) among GFR values of HC group and CKD group, according to Model C, showed a significant difference ($p=0.001$) between the two groups of subjects. The t-test analysis ($p<0.05$) among GFR values of HC group and CKD group, according to Model D, showed a significant difference ($p=0.004$) between the two groups of subjects.

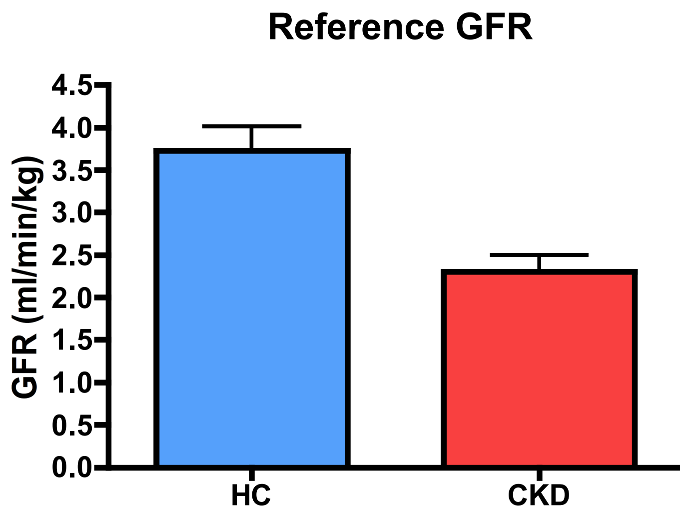

Figure 1: t-test analysis between GFR of HC group and CKD group. GFR was significantly different (p=0.003) between HC and CKD patients. HC subjects showed a mean GFR of 3.25 ± SE 0.27 ml/min/kg while CKD subjects a value of 2.30 ± SE 0.19.

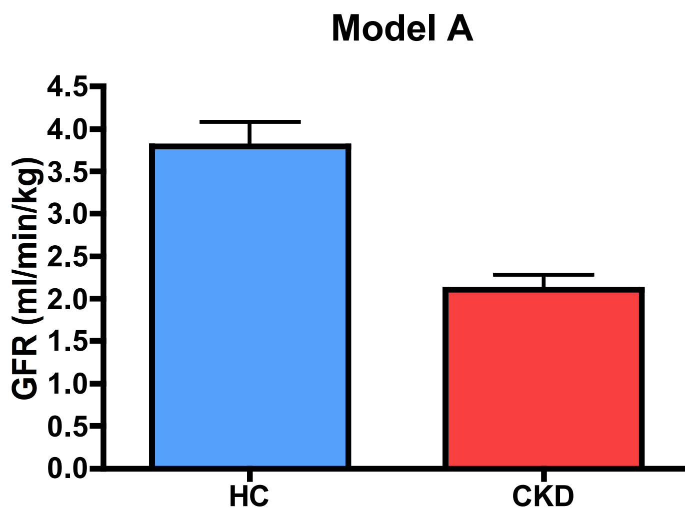

Figure 2: t-test analysis between GFR of HC group and CKD group, according to Model A. GFR was significantly different (p=0.0005) between HC and CKD patients. HC subjects showed a mean GFR of 3.32 ± SE 0.47 ml/min/kg while CKD subjects a value of 2.11 ± SE 0.17.

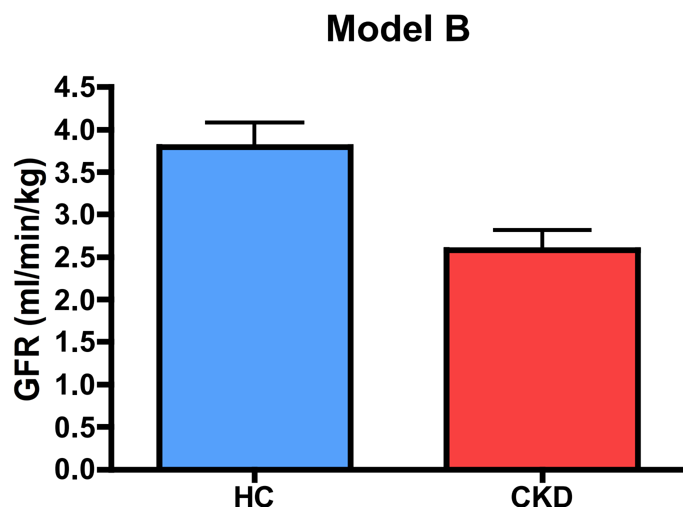

Figure 3: t-test analysis between GFR of HC group and CKD group, according to Model B. GFR was significantly different (p=0.01) between HC and CKD patients. HC subjects showed a mean GFR of 3.28 ± SE 0.38 ml/min/kg while CKD subjects a value of 2.57 ± SE 0.23.

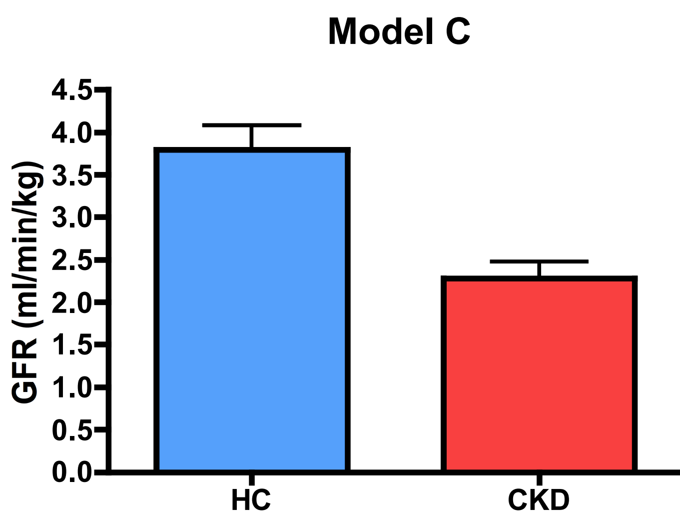

Figure 4: t-test analysis between GFR of HC group and CKD group, according to Model C. GFR was significantly different (p=0.001) between HC and CKD patients. HC subjects showed a mean GFR of 3.08 ± SE 0.41 ml/min/kg while CKD subjects a value of 2.28 ± SE 0.19.

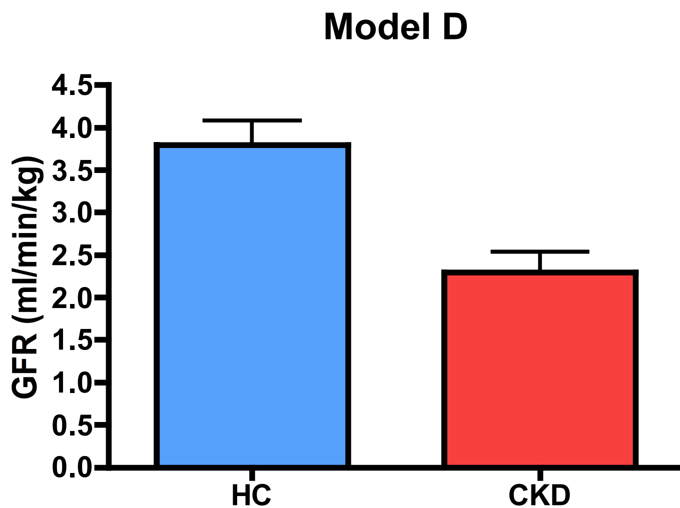

Figure 5: t-test analysis between GFR of HC group and CKD group, according to Model D. GFR was significantly different (p=0.004) between HC and CKD patients. HC subjects showed a mean GFR of 3.43± SE 0.39 ml/min/kg while CKD subjects a value of 2.45± SE 0.26.

Pearson analysis in clinically healthy (HC) cats

Pearson correlation test ($p<0.05$) between GFR values obtained by reference method and Model A (5' – 30' – 1 and 4 hours) showed a positive linear correlation ($p<0.0001$) characterized by a value of R^2 of 0.92 and a Pearson r of 0.95. Similarly, Bland-Altman analysis reported no significant clinical difference (bias=0.10) in average versus difference between GFR values assessed through reference method and Model A. Pearson correlation test ($p<0.05$) between GFR values obtained by reference method and Model B (5' – 30' – 1 and 2 hours) showed a positive linear correlation ($p<0.0001$) characterized by a value of R^2 of 0.95 and a Pearson r of 0.97. Similarly, Bland-Altman analysis reported no significant clinical difference (bias=-0.39) in average versus difference between GFR values assessed through reference method and Model B. Pearson correlation test ($p<0.05$) between GFR values obtained by reference method and Model C (5' – 1 – 2 and 4 hours) showed a positive linear correlation ($p<0.0001$) characterized by a value of R^2 of 0.88 and a Pearson r of 0.94. Similarly, Bland-Altman analysis reported no significant clinical difference (bias=-0.04) in

average versus difference between GFR values assessed through reference method and Model C. Pearson correlation test ($p<0.05$) between GFR values obtained by reference method and Model D (5'- 30' – 1 hour) showed a positive linear correlation ($p<0.0001$) characterized by a value of R^2 of 0.83 and a Pearson r of 0.91. Similarly, Bland-Altman analysis reported no significant clinical difference (bias=-0.80) in average versus difference between GFR values assessed through reference method and Model D.

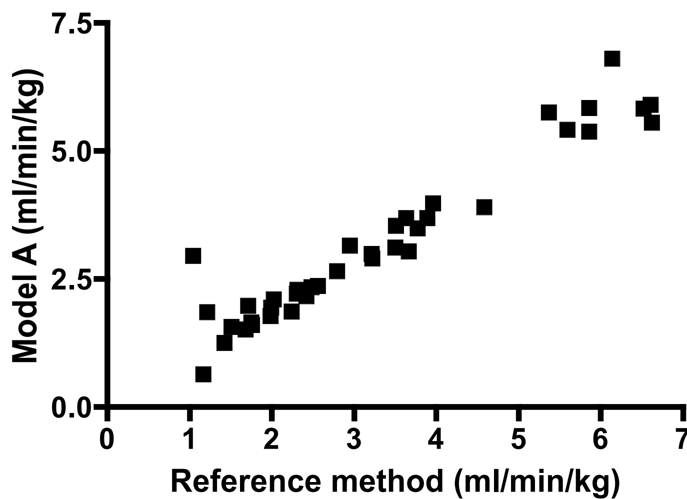

Figure 6: Pearson correlation test between GFR assessed through reference method and Model A (5'-30'-1 and 4 hours). Positive linear correlation ($p<0.0001$) characterized by R^2 of 0.92.

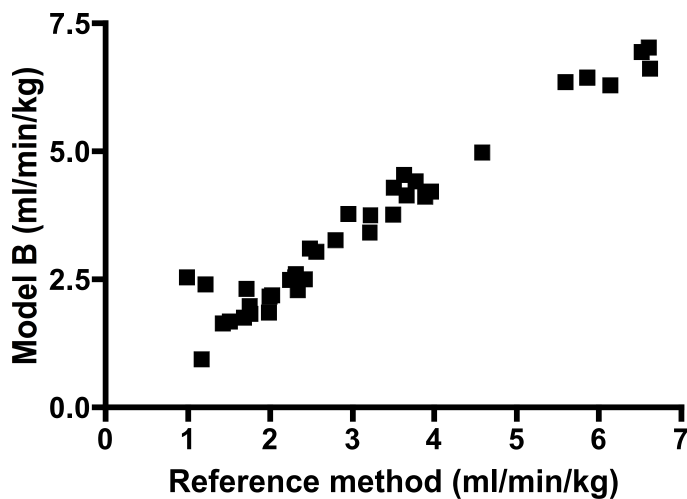

Figure 7: Pearson correlation test between GFR assessed through reference method and Model B (5'-30'-1 and 2 hours). Positive linear correlation ($p<0.0001$) characterized by R^2 of 0.95.

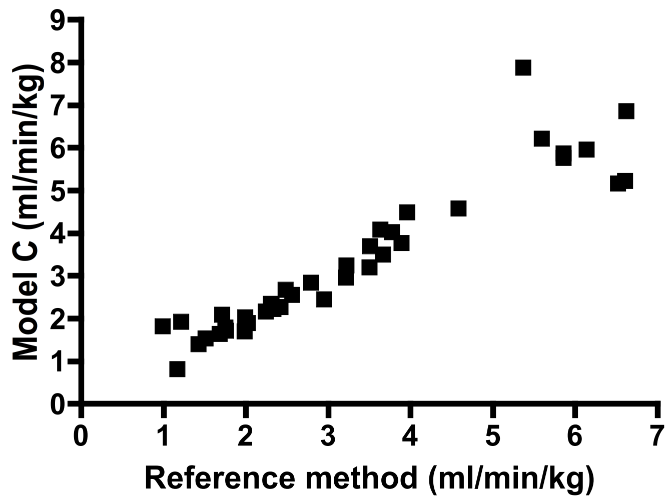

Figure 8: Pearson correlation test between GFR assessed through reference method and Model C (5'- 1 - 2 and 4 hours). Positive linear correlation ($p<0.0001$) characterized by R^2 of 0.88.

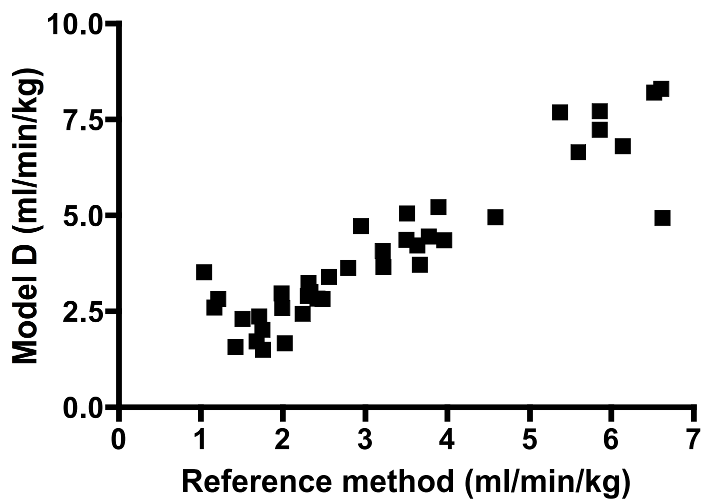

Figure 9: Pearson correlation test between GFR assessed through reference method and Model D (5'- 30' and 1 hour). Positive linear correlation ($p<0.0001$) characterized by R^2 of 0.83.

Bland-Altman analysis in HC

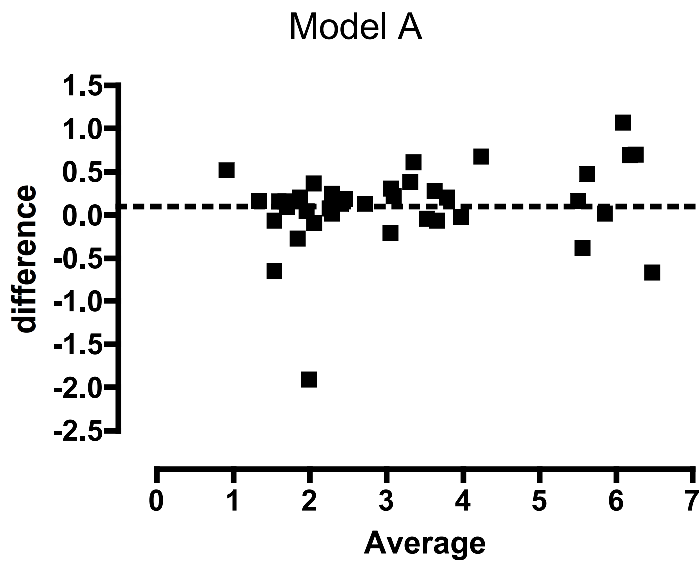

Figure 10: Bland-Altman plot of difference versus average of GFR assessed through reference method and through Model A. Bias of difference versus average was not clinically significant (0.10).

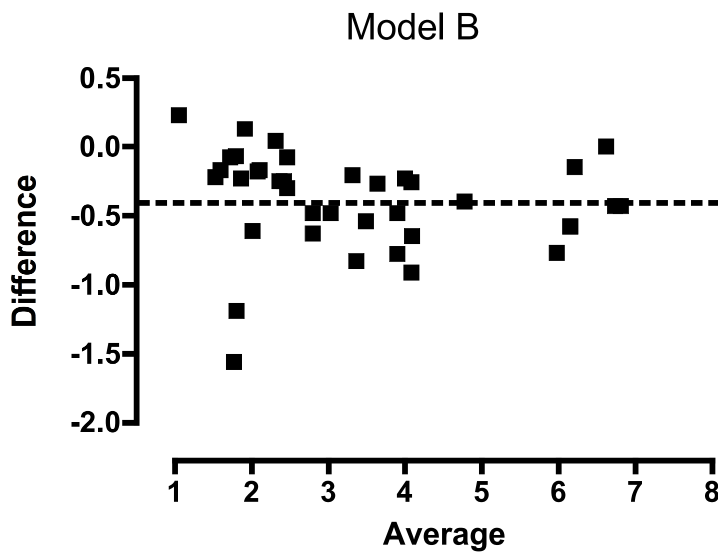

Figure 11: Bland-Altman plot of difference versus average of GFR assessed through reference method and through Model B. Bias of difference versus average was not clinically significant (-0.39).

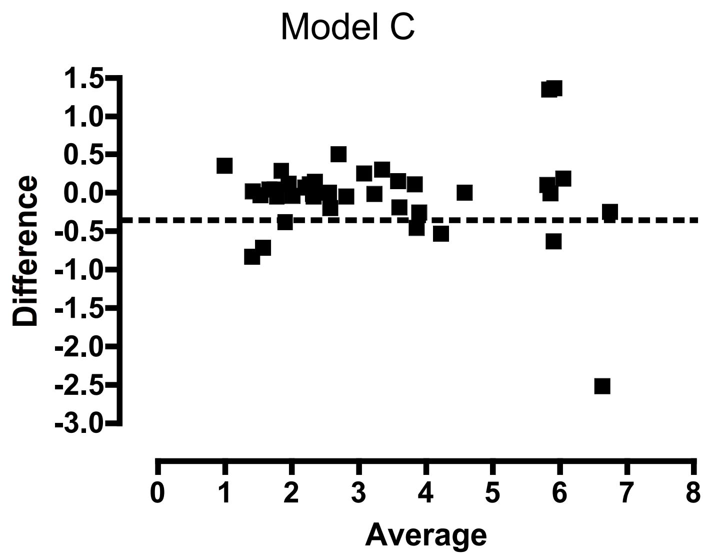

Figure 12: Bland-Altman plot of difference versus average of GFR assessed through reference method and through Model C. Bias of difference versus average was not clinically significant (-0.04).

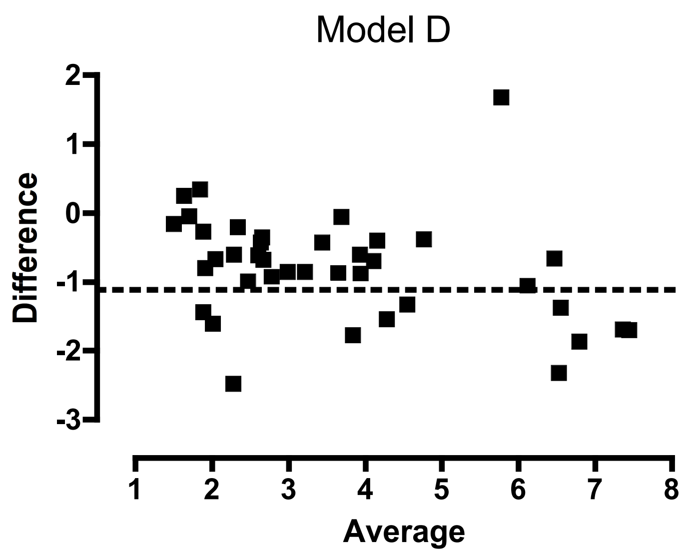

Figure 13: Bland-Altman plot of difference versus average of GFR assessed through reference method and through Model D. Bias of difference versus average was not clinically significant (-0.80).

Pearson analysis in chronic kidney disease (CKD) cats

Pearson correlation test ($p<0.05$) between GFR values obtained by reference method and Model A (5' – 30' – 1 and 4 hours) showed a positive linear correlation ($p<0.0001$) characterized by a value of R^2 of 0.92 and a Pearson r of 0.97. Similarly, Bland-Altman analysis reported no significant clinical difference (bias=0.20) in average versus difference between GFR values assessed through reference method and Model A. Pearson correlation test ($p<0.05$) between GFR values obtained by reference method and Model B (5' – 30' – 1 and 2 hours) showed a positive linear correlation ($p<0.0001$) characterized by a value of R^2 of 0.95 and a Pearson r of 0.99. Similarly, Bland-Altman analysis reported no significant clinical difference (bias=-0.31) in average versus difference between GFR values assessed through reference method and Model B. Pearson correlation test ($p<0.05$) between GFR values obtained by reference method and Model C (5' – 1 – 2 and 4 hours) showed a positive linear correlation ($p<0.0001$) characterized by a value of R^2 of 0.98 and a Pearson r of 0.99. Similarly, Bland-Altman analysis reported no significant clinical difference (bias=0.05) in average versus difference between GFR values assessed through reference method and Model C. Pearson correlation test ($p<0.05$) between GFR values obtained by reference method and Model D (5'- 30' – 1 hour) showed a positive linear correlation ($p<0.0032$) characterized by a value of R^2 of 0.59 and a Pearson r of 0.77. Similarly, Bland-Altman analysis reported no significant clinical difference (bias=-0.18) in average versus difference between GFR values assessed through reference method and Model D.

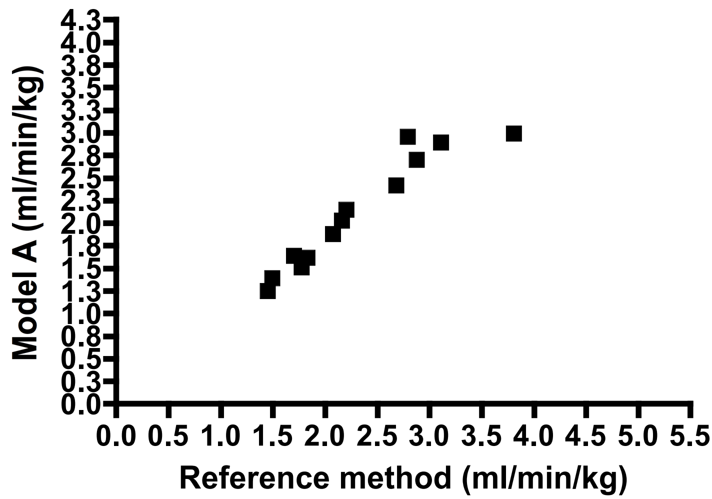

Figure 14: Pearson correlation test between GFR assessed through reference method and Model A (5'-30'-1 and 4 hours). Positive linear correlation ($p<0.0001$) characterized by R^2 of 0.97.

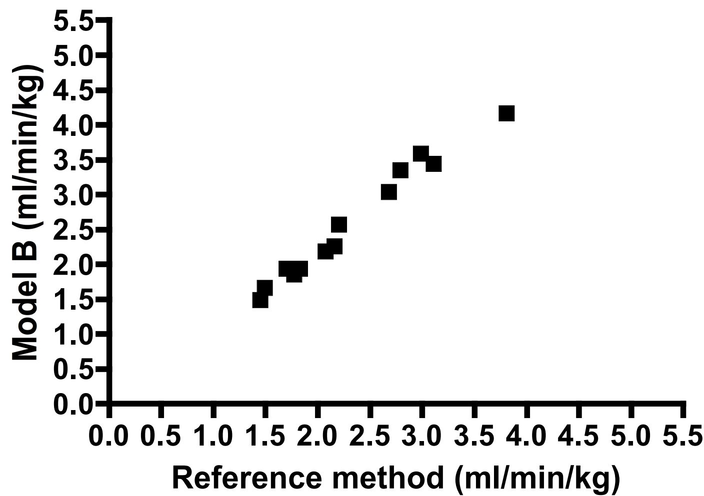

Figure 15: Pearson correlation test between GFR assessed through reference method and Model B (5'-30'-1 and 2 hours). Positive linear correlation ($p<0.0001$) characterized by R^2 of 0.98.

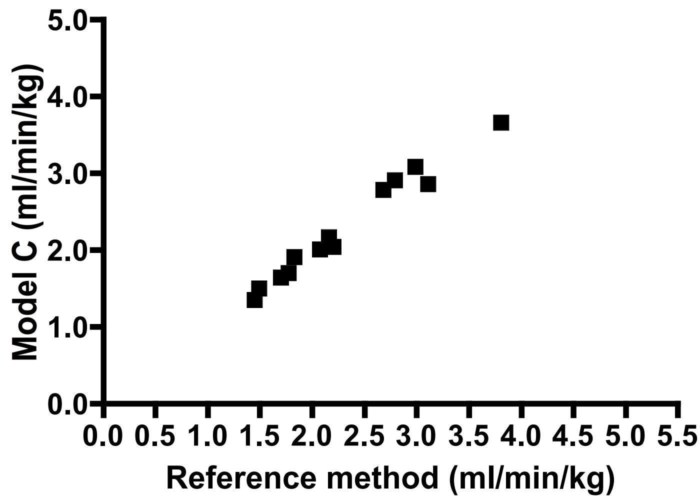

Figure 16: Pearson correlation test between GFR assessed through reference method and Model C (5'- 1 - 2 and 4 hours). Positive linear correlation ($p<0.0001$) characterized by R^2 of 0.98.

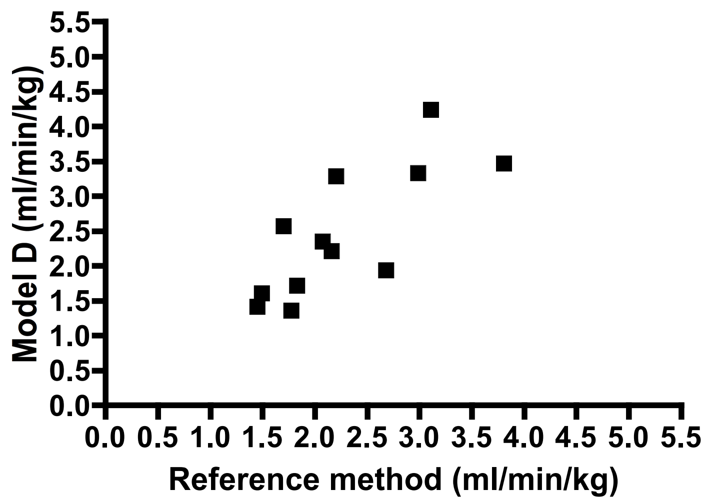

Figure 17: Pearson correlation test between GFR assessed through reference method and Model D (5'- 30' and 1 hour). Positive linear correlation ($p<0.0032$) characterized by R^2 of 0.59.

Bland-Altman analysis in CKD

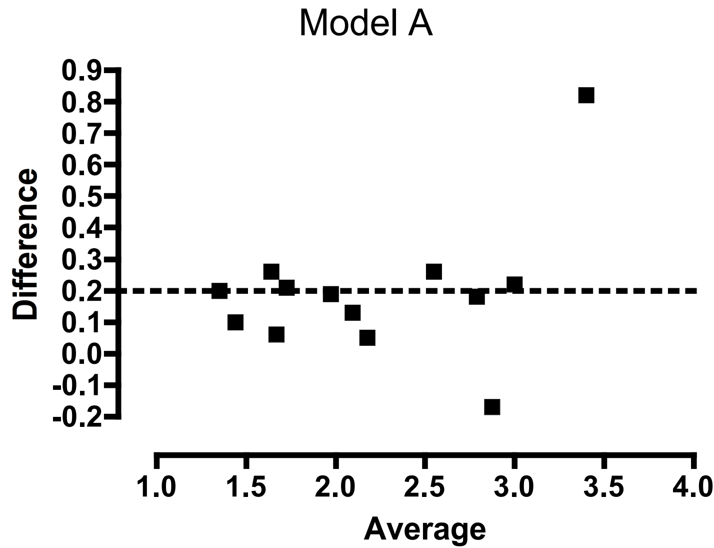

Figure 18: Bland-Altman plot of difference versus average of GFR assessed through reference method and through Model A. Bias of difference versus average was not clinically significant (0.20).

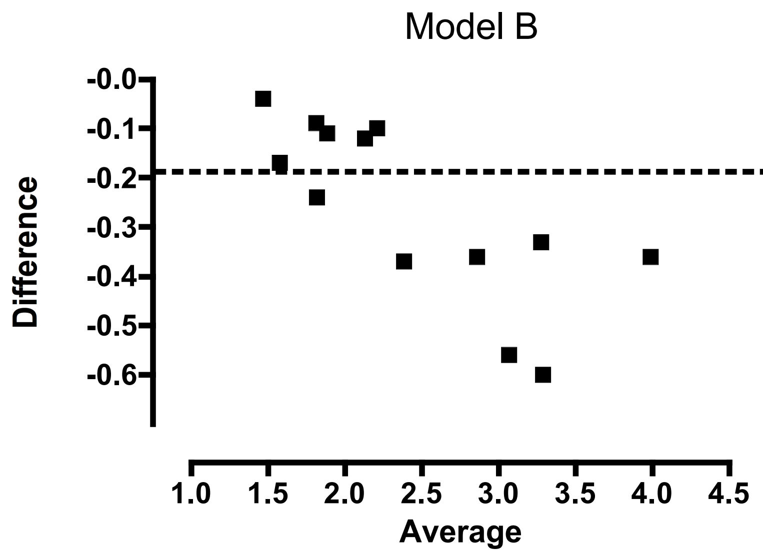

Figure 19: Bland-Altman plot of difference versus average of GFR assessed through reference method and through Model B. Bias of difference versus average was not clinically significant (-0.31).

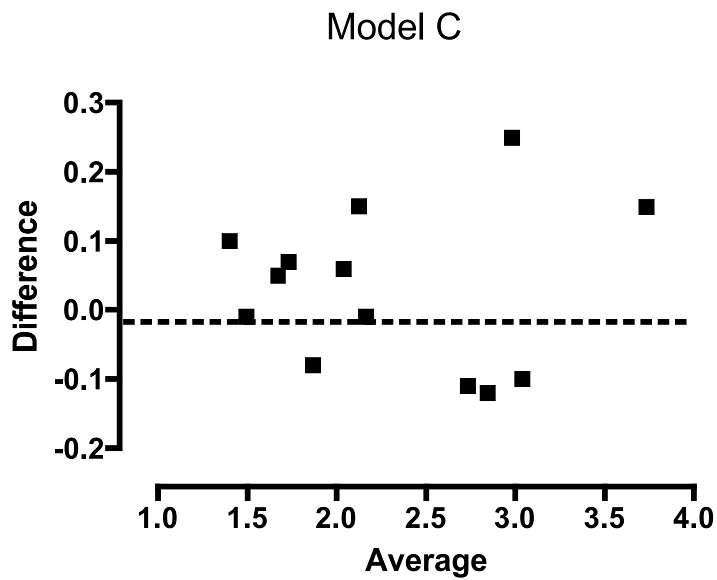

Figure 20: Bland-Altman plot of difference versus average of GFR assessed through reference method and through Model C. Bias of difference versus average was not clinically significant (0.05).

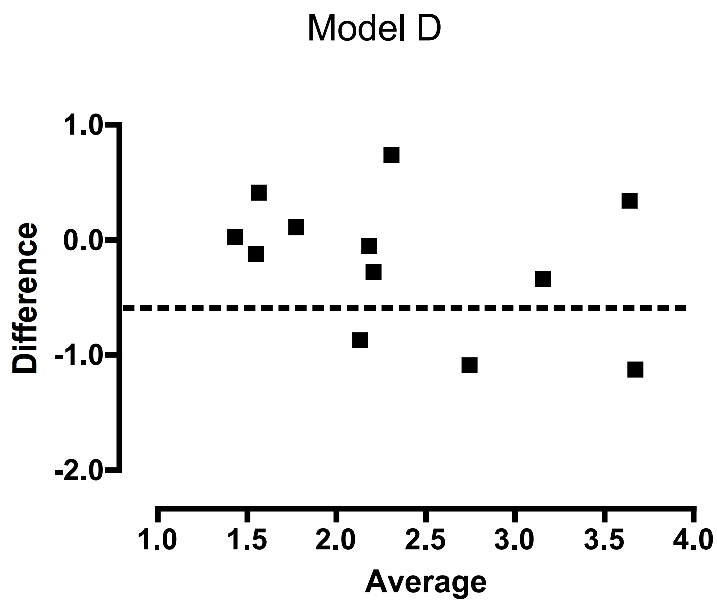

Figure 21: Bland-Altman plot of difference versus average of GFR assessed through reference method and through Model D. Bias of difference versus average was not clinically significant (-0.18).

CHAPTER 6: DISCUSSION

CKD represents a complex clinical syndrome with an even more increasing prevalence in small animal population. Recent reports showed, in fact, that CKD plays an important epidemiological role in canine and feline population. Epidemiological studies concern only IRIS stages 2, 3 and 4 in which plasma values of creatinine are above the reference range and, at present, no data about the prevalence of the disease in IRIS stage 1 are reported. Both in dogs and cats, IRIS 1 is an asymptomatic and subclinical stage characterized by plasma levels of creatinine within the normal range, which cannot be detected without using GFR. The importance of early diagnosis in slowing down the progression of CKD has been widely demonstrated both in human and in veterinary medicine[1]. CKD is inexorably doomed to develop end-stage renal disease (ESRD). At this point just haemodialysis and renal transplantation are effective treatments. Because of the high costs and low availability of these treatments, the early onset of a conservative nutritional and medical therapy shows a dramatic importance in postponing ESRD. Unfortunately, although GFR is universally considered the gold standard test to evaluate overall renal function, its use in veterinary clinical practice is still uncommon, due to technical difficulties, high number of samples and low availability of markers. Plasma clearance methods seem to be easier to be performed compared to urinary clearance ones and can represent an attractive way of GFR determination[2]. Iohexol is an iodinate contrast medium that has been shown to be a good alternative to inulin and radioactive tracers in human, pig, horse, dog, donkey and cat[2,3,4,5]. The advantages of iohexol, over other markers, include lack of radiation hazard and associated radiation protection measures, easy commercial availability and assay, and low price[2]. The development of an accurate three-hour and four-sample GFR method in canine patients, by using plasma clearance of iohexol, encouraged us to evaluate different sampling schemes even in cats. The low compliance of feline patients, compared to dogs, led us to develop blood-sampling combinations with the smallest number of samples. The present study presented a fast, accurate and relatively simple method for GFR determination in feline

patients. Currently available HPLC systems to determine plasma iohexol concentration were proven to give reliable data, but they still present some disadvantages to be routinely used for the early diagnosis of CKD in a clinical setting. The method investigated in the present study combined an easy sample preparation and a rapid HPLC run with a simple mobile phase. Analysis were performed with inexpensive, not dangerous and readily available chemicals. The robustness of the method made easy for operators to learn the technique and to generate reproducible results. The method indeed showed itself to be very economical, with an approximate cost per sample of less than two Euros for supplies and materials. In fact, a single analytical column, under the assay condition, lasted for the entire period of method validation and clinical study. Furthermore, the stability test indicated that plasma samples can be frozen or sent by mail, and this would be attractive for general practitioners, who could send serum samples to a reference laboratory. This HPLC method required very small volumes of plasma samples (50 μl). Such limited amounts of plasma may be a significant advantage in a feline clinical setting, especially when anaemic or dehydrated animals are involved. At the dosage (64.7 mg/kg) used in this study, iohexol can be safely utilized even in debilitated or severely azotemic subjects[6,7]. Furthermore, no one of the enrolled cats showed immediate or subsequent side effects. In the the Author's experience the use of two intravenous catheters (one utilized for iohexol injection and the other one to take blood samples) increased the compliance of owners and patients and should be preferred to several blood samples. The short time required for the GFR test and the small volume of blood needed made the use of an intravenous catheter to take blood easily. All the four analyzed models (Model A, Model B, Model C and Model D) offered a dramatic simplification of reference method, in terms of time and number of blood samples. Accuracy of GFR determination was not compromised by a reduction of the number of plasma samples. Pearson correlation analysis ($p<0.05$) between reference method and each of the simplified models showed a positive linear correlation, with very high values of R^2 and Pearson r. Similarly, Bland-Altman analysis of difference versus average reported very low values of bias, which showed a clinically irrelevant difference between reference method and each simplified model. Mean GFR value of clinically healthy subjects (mean ± SEM, 3.40 ± 0.29 ml/min/kg) was in agreement with previously reported data for plasma clearance of iohexol in feline patients[8] and it differed significantly from mean GFR of CKD patients, both using reference

method (p=0.003) and simplified Model A (p=0.0005), Model B (p=0.01), Model C (p=0.001) and Model D (p=0.004). In CKD cats, GFR showed a mean value of 2,38±0.23 ml/min/kg, with a minimum of 1.36 ml/min/kg and a maximum of 3.47 ml/min/kg. Clinically healthy cats, instead, presented a wider range of GFR with a mean value between a minimum of 1.21 ml/min/kg and a maximum of 8.62 ml/min/kg. No one of CKD patients showed a GFR above 3.50 ml/min/kg. According to the guidelines of the International Renal Interest Society (IRIS), IRIS 1 patients present plasma values of creatinine within the normal range and are generally asymptomatic. So, there is no way to identify these patients without using GFR. UP/UC and systolic blood pressure (included into IRIS guidelines) only represent risk factors of CKD and they are not sufficient for a correct identification of IRIS 1 subjects[9]. According to these considerations, it was supposed that cats enrolled in the present study as clinically healthy, with a GFR below 2.00 ml/min/kg, may have been in an early stage of CKD. This hypothesis was suggested by the fact that the group of cats belonging to the HT group and with a GFR below 2.00 ml/min/kg showed a plasma iohexol decay pattern more similar to CKD than HT subjects. This group was composed by 11 subjects with a mean GFR of 1.14 ± 0.09 ml/min/kg, within a minimum of 1.04 ml/min/kg and a maximum of 1.99 ml/min kg. Their mean GFR appeared significantly more related to CKD than to HT group. These data led us to the conclusion that this group of subjects was very probably composed by asymptomatic IRIS 1 patients. Although a wider number of subjects would be recommended for an accurate establishment of GFR range, present data and reference reports strongly suggest that feline patients with a GFR below 2.00 ml/min/kg should be considered in an early stage of CKD. The use of a seven-sample method to estimate plasma clearance of a tracer is an extremely accurate way of performing curve fitting with nonlinear regression analysis, but it is too time-consuming and expensive and it could be excessively stressful for feline patients. Furthermore, the high number (seven) of blood samples required is too cumbersome to be used in a clinical setting. Limited sampling strategies for plasma clearance procedures were investigated extensively in humans[10], and more recently in animals[11,2], to establish a quick, inexpensive, and clinically accurate value of GFR. All simplified models (Model A, Model B, Model C and Model D), that were taken into consideration in the present study, showed a high accuracy in determining GFR, both in healthy and in CKD patients, and represented a significant simplification of the reference method. Anyway, among different

models, Model D (5'- 30' -1 hour) showed the best solution because it combined an accurate GFR determination with a very quick and easy to perform method. The extremely good correlation between Model D and reference method was demonstrated by high levels of R^2 and Pearson P, both in HC (R^2=0.83, $p<0.0001$) and CKD patients (R^2=0.59, $p<0.0032$).

In conclusion, the present study validated a safe, simple and accurate three-sample HPLC method (5' – 30' – 1 hour) for the determination of GFR through the plasma clearance of iohexol in feline patients. This model represents an attractive and cheap alternative to cumbersome plasma clearance methods, with a dramatic applicatory potential in different clinical settings. The accuracy of HPLC analysis, the possibility to mail plasma samples to a referring laboratory and the high compliance of this method, would lead general practitioners to an easier diagnosis of subclinical stages of CKD and to a better management of the disease. After owners' informed consent, 53 clinically healthy and 14 CKD cats have been submitted to an eight-hour clearance study.

CHAPTER 7: CALCIUM-PHOSPHATE METABOLISM IN CKD: TOTAL CALCIUM, IONIZED CALCIUM, CORRECTED CALCIUM AND CA X P PRODUCT

Researches in human medicine have been focusing on the importance of mineral metabolism disorders and metabolic bone diseases in the progression of CKD[1,2,3]. For this reason a new classification of CKD has been proposed by the Kidney Disease Improving Global Outcomes (KDIGO), in order to consider even bone and mineral disorders. The new Chronic Kidney Disease – Bone and Mineral Disorders (CKD-BMD) staging has been accepted by the nephrology community and should be taken into consideration for a more accurate classification of the CKD patient. Recent epidemiologic and clinical acquisitions reported an increased mortality risk in the CKD population affected by disturbances of mineral metabolism. High levels of serum phosphate, enhanced by elevated blood calcium concentrations, would be responsible for an amplification of vascular calcification and would be related to an increased risk of cardiovascular mortality[1,2,3,4]. CKD patients are often affected by secondary hyperparathyroidism (SHPT) as a consequence of a condition of hyperphosphatemia, hypocalcemia and decreased concentration of 1,25-dihydroxy-vitamin D3. SHPT in CKD shows a complex pathogenesis which involves phosphate retention, hyperphosphatemia, low serum ionized calcium (iCa), elevated parathyroid hormone (PTH), vitamin D deficiency, intestinal calcium malabsorption, reduction of vitamin D and calcium-sensing receptors in the parathyroid glands and altered mRNA-binding protein modulating PTH transcripts[16]. Serum PTH concentration depends on both the release of PTH stored into the parathyroid glands and on the synthesis of new hormone. PTH can be rapidly released by the parathyroid glands in hypocalcemia through the interaction between iCa and calcium sensing receptors (CaSR)[16]. CaSR are located on the surface of parathyroid cells and regulate PTH release in response to changes in the concentration of iCa. In physiologic conditions, as the serum calcium concentration falls, stored PTH is released into the blood stream. PTH increases the renal tubular calcium absorption through activation of the kidney calcium channels TRPV5 and it stimulates the activity of the renal-25-hydroxy-vitamin-D1-alpha hydroxilase leading to increment of the plasma concentration of 1-25-$(OH)_2D_3$. The action of

$1\text{-}25\text{-}(OH)_2D_3$ promotes an increased absorption of calcium from the small intestine by stimulating a transcriptional regulation of calcium channels TRPV6. These actions have the combined effect of raising the concentration of serum calcium. Alternatively, an increase in iCa concentration determines an inhibiting activity of CaSR on PTH release. CaSR are also present in thyroid tissue where they have been shown to regulate calcitonin release. Calcitonin acts mostly by inhibiting osteoclastic activity and, therefore, the release of calcium from bone[18]. The biological importance of CaSR in the homeostasis of iCa has been demonstrated by discovering familial hypercalcemic or hypocalcemic conditions due to a inactivating or activating mutation of CaSR. Transcription of CaSR is not influenced by iCa but by the concentration of vitamin D in the parathyroid glands, kidneys and thyroid C cells. CKD patients show reduced amounts of CaSR as a result of parathyroid hyperplasia and low serum levels of vitamin D. The reduction in CaSR concentration in the parathyroid glands attenuates the responsiveness of the gland to iCa and contributes to develop SHPT[16]. At the same time a reduction in number of vitamin D receptors (VDR) modifies the transcription of CaSR and PTH. In addition, CKD subjects can present changes in the amount of mRNA encoding PTH binding proteins. SHPT is a complex metabolic condition due to the onset of multiple abnormalities that are able to enhance the rate of PTH release and synthesis[16]. Homeostasis of calcium and phosphate is tightly linked and this relationship is best illustrated in diseases like CKD and SHPT[18]. The attempt to avoid SHPT led to the formulation of specific guidelines, which try to prevent an overstimulation of the parathyroid glands[6]. A strict relationship between a long-term exposure to high serum levels of calcium and phosphate and an increased risk of mortality has been widely documented and it is considered the main target of a conservative therapy. In human CKD patients, in fact, cardiovascular complications represent the most frequent cause of mortality. It is not clear if there is a direct causal relationship between bone health and calcification or if this is a common calcification pathway as part of the CKD-MBD disease process[17]. A calcium- phosphate imbalance can increase the risk of vascular calcification in end stage renal disease (ESRD) patients. The ongoing debate about the predominant origin of the vascular calcifications in this category of patients is trying to clarify if they are caused by an atherosclerotic or an arteriosclerotic injury or both. Although the differentiation between atherosclerosis and arteriosclerosis is essential for a correct prognosis and treatment, it can be hard to differentiate even using advanced

imaging techniques[7,8,9]. Both processes are characterized by a vessel calcification, which shows a different pathogenesis.

Atherosclerosis is determined by a patchy calcification of the arterial intima around lipid deposits and it can be a common report of the Aorta and other large arteries in hyperlipidaemia and in advanced age. In human patients atherosclerosis begins as early as the second decade of life and small aggregates of crystalline calcium have been documented within the lipid core of coronary artery plaques of young adults[10]. Calcium phosphate represents the predominant form in arterial calcium deposits and it is secreted in a similar way to what found in osteogenesis and bone remodelling. Vesicles containing calcium phosphate are secreted by arterial wall cells (vascular smooth muscle cells and pericytes) as matrix vesicles do from condrocytes during bone developing[8]. Microvascular endothelial dysfunctions seem to play a fundamental role in the pathogenesis of atherosclerosis in renal failure. Glomerular endothelial injuries may develop in the early stages of renal disease hypertension, before the onset of blood pressure elevation. Endothelial dysfunction presents multifactor causes that are strictly related, like increased oxidative stress, dyslipidemia and inflammation. CKD patients become progressively malnourished showing low levels of albumin, prealbumin and transferrin, that have been supposed to be activating factors of inflammation. Anyway, the often concomitant presence of diabetes and hypertension makes difficult to understand the direct role of early stage renal failure in promoting inflammation. CKD dyslipidemia is characterized by a progressive increase of high-density lipoproteins (HDL) and decrease of low density lipoproteins (LDL). In particular, the synthesis of Hepatic apolipoprotein A-I reduces and Apolipoprotein C-III (competitive inhibitor of liporpotein lipase) increases, resulting in serum accumulation of triglycerides. The relative low serum concentration in HDL is responsible for a reduction in antioxidant power and in endothelial protective effect towards proinflammatory cytokines[10]. Oxidative stress may involve activation of reduced nicotinamide adenine dinucleotide (NAD(P)H) oxidase, xanthine oxidase, uncoupled endothelial NO synthase, myeloperoxidase (MPO) and mitochondrial oxidase. Increased production of reactive oxygen species (ROS) by uncoupled endothelial NO synthase as well as their reduced inactivation by the antioxidant system may accelerate atherosclerosis. Moreover the activation of the renin-angiotensin system, frequently reported in CKD patients, can stimulate NAD(P)H oxidase, leading to generation of superoxide anion

able to cause endothelial injuries. The interaction between angiotensin II and AT_1 receptor stimulates production of ROS and other enzyme systems that determine up-regulation of inflammatory mediators such as cytokines, chemokines, adhesion molecules, plasminogen activator inhibitor 1 and superoxide scavenging of nitric oxide (NO). Reduced bioavailability of NO is considered one of the main factors involved in CKD-associated endothelial dysfunction, due to increased oxidative stress in the vascular wall[15]. Asymmetric dimetyl arginine (ADMA), competitive inhibitor of NO synthase, may decrease the synthesis of NO. High plasma levels of ADMA are associated with endothelial injury and atheroslerosis and they have been reported predictors of cardiovascular complications and mortality in ESRD[10]. Arteriosclerosis, instead, is characterized by a metabolite-induced calcification of the arterial media of large vessels in the absence of lipid concretions[10]. Increased serum calcium-phosphate levels can cause vascular changes that lead to an up-regulation of the osteogenic differentiation of vascular smooth muscle cells. The concentric media thickening of muscular arteries is responsible for a not age-related vascular stiffening which has been shown also in ESRD children and animal models[7,8,9]. The arterial stiffening occurring in arteriosclerosis involves an active calcium phosphate transport through the action of cotransporter PiT-1. The stimulation of PiT-1 activity is determined by both a phenotypic switch of vascular smooth muscle cells into osteoblast-like cells, as a consequence of high intracellular concentrations of calcium and phosphate, and by a functional impairment of calcification inhibitors[10]. An in vitro study demonstrated that elevated concentrations of calcium or phosphate promote calcification of human vascular smooth muscle cells. This process was started by release of membrane-bound matrix vesicles and apoptotic bodies. Vesicles released by cells exposed to calcium and phosphate calcified to an important degree, but those released in the presence of serum were just minimally calcified and were found to contain calcification inhibitors[11]. Arteriosclerosis appears as a result of an imbalance between the serum concentration of calcification promoters and inhibitors. In human medicine the following inhibitors of calcification were identified: fetuin-A, MGP, Osteoprotegerin, BMP-7 and pyrophosphate (PPi). Fetuin-A forms stable colloidal spheres with calcium and phosphate and becomes part of a high molecular mass complex containing calcium, phosphate and matrix GLA protein (MGP). A reduction in Fetuin-A serum concentration was related to an increased risk of vascular calcification. MGP belongs to a family of proteins that requires a vitamin K-

dependent gamma carboxylation for its biological activity and the gamma carboxylated form is carried in plasma by Fetuin-A. MGP is a modulator of the activity of bone morphogenetic protein-2 (BMP-2). Mice lacking MGP developed spontaneous calcification of arteries and cartilage. On the other hand, high levels of MGP were found in the vicinity of atherosclerotic plaques and were associated with in vitro calcification of smooth muscular cells. The ability of MGP to reduce or increase calcification seems to depend to availability of BMP-2. MGP inhibits calcification of calcifying vascular cells with high relative concentration of BMP-2, while it stimulates calcification with low relative levels of BMP-2[10,12]. Osteoprotegerin is a modulator of osteoclast activation which mechanism of action has not been clearly elucidated. A deficiency of Osteoprotegerin in mice was related to an increased vascular calcification, while high serum concentration, especially when associated with elevated levels of C-reactive protein (CRP), correlates with increased calcification and mortality in haemodialysis patients. ESRD subjects are generally characterized by elevated serum concentrations of Osteoprotegerin[10,13,14]. PPi is synthesized by the nucleotide pyrophosphatase phospho-diesterase-1 and its deficiency is associated with a proliferation of altered vascular smooth muscle cell phenotype and increased calcification. Calcium phosphate deposits may also result from enhanced activity of the membrane-bound tissue-nonspecific alkaline phosphatase, which degrades PPi to P. PPi deficiency can occur in ESRD patients as a consequence of haemodialysis removal. BMP-7 is part of the BMP family of regulators of bone formation. Despite BMP-2 and BMP-4, BMP-7 is an inhibitor of vascular calcification, which is expressed mainly in the kidney. BMP-7 causes up regulation of a alpha-smooth muscle actin through induction of p21 and up regulation of Smad 6 and 7. A reduction in BMP-7 affects bone metabolism, determining an increase in serum phosphate, which induces phenotypic changes in vascular smooth muscle cells and metastatic calcification[10]. Although the debate about the role of atherosclerosis and artherosclerosis on cardiovascular complications in CKD patients is still open, a recent study[7] did not find any correlation among long-term calcium phosphate exposure in ESRD subjects and intima-media thickness (IMT) and ankle-brachial index (ABI), both early markers of atherosclerosis.

Finally, there is also a specific dialysis-related type of vascular calcification called calciphylaxis or uremic arteriopathy, which is characterized by diffuse calcification of the tunica media of small to medium arteries and arterioles. Patients affected by calciphylaxis

show even intimal proliferations and trombosis that can lead to skin ulcers and life-threatening skin necrosis or gangrene. Calciphylaxis develops in a condition of elevated serum calcium – phosphate product (Ca x P) without the presence of an active osteogenic process and it differs from other forms of skin calcification that do not affect blood vessels.

Kidneys play a key role in maintaining calcium and phosphate homeostasis through different and closely integrated processes. Despite calcium is filtered by the glomerulus, less than 3% of the filtered load is eliminated through urine. Calcium is, in fact, submitted to an intense tubular reabsorption, which occurs both passively and actively. The majority of calcium reabsorption is passive and it occurs in the proximal tubule. Within the proximal tubule calcium strictly follows sodium movements. Active reabsorption takes place in the thick ascending limb of the loop of Henle, in the distal convolute tubule and in the connecting tubule. In the thick ascending limb of the loop of Henle calcium is associated to the sodium-potassium pump. The potassium movement towards tubule lumen determines a concentration gradient, which facilitates calcium reabsorption through the paracellular protein paracellin. In the distal convoluted tubule and in the connecting tubule calcium reabsorption follows different steps. Firstly calcium enters cells through apical TRPV5 calcium channel. The binding between calcium and the binding protein calbindin-D28k is responsible for a cytosolic transfer to the basolateral membrane. Calcium can then re-enter the extracellular compartment through the action of the Na^{+}/Ca^{2+} exchanger (NCX1) and the Ca^{2-} pump (PMCAlb). TRPV5 activity, trafficking and endosomal recycling are regulated by several proteins and ions, among which klotho, tissue kallikrein factor, pH, estrogens and intracellular Ca^{2-} concentration. Klotho is an antiaging hormone which stimulates TRPV5 activity, leading to an increase of calcium intake. Tissue kallikrein is synthesized in the distal nephron. After its release into tubular filtrate tissue, kallikrein binds to kinin receptors and, through a protein kinase C pathway, increases TRPV5 expression resulting in augmented calcium reabsorption. PH can directly interact with TRPV5 receptors by regulating their expression in response to conditions of alkalosis or acidosis. This mechanism is supposed to be due to a pH-controlled insertion of TRPV5 containing vesicles in the apical plasma membrane, which is able to stimulate hypo- or hypercalciuria according to pH modifications. Estrogens have a stimulating effect on TRPV5 receptors, which is vitamin vitamin D_3 independent. Finally, TRPV5 can be modulated by calcium itself, which is responsible for a negative feedback towards the

receptors[18]. The majority of filtered phosphate is reabsorbed in renal proximal tubule epithelial cells through a sodium-coupled transport. This process is driven by basolateral Na-K-ATPases and apical Na/P_i type II cotransporters. Although the Na/P_i II cotransporters have three isoforms, just type IIa and type IIc are present in the renal proximal tubular cell apical membrane, while type IIb is primary located in the small intestine. Studies in mice demonstrated that the majority of renal phosphate reabsorption is due to type IIa cotransporter, while type IIc plays a lesser role. Anyway recent acquisitions in human medicine underlined a certain importance of cotransporter type IIc. Renal phosphate reabsorption is influenced by different hormonal and non-hormonal factors, that can interact with Na/P_i cotransporters. PTH leads to an increased urinary excretion of phosphate by reducing the quantity of type IIa cotransporters in the apical brush border membrane of renal proximal tubule cells, through a PTH-induced endocytosis. In a similar way, high dietary phosphate intake is responsible for a down-regulation of type II cotransporters. On the contrary, low dietary phosphate intake stimulates increased expression of these cotransporters both through enhanced stability and increased insertion at the apical membrane. Elevated serum phosphate concentration also inhibits the synthesis of vitamin D_3. In addition to PTH and phosphate there are other factors, termed phosphotonins, able to modulate renal reabsorption of phosphate. The phosphotonin FGF-23 has been showed to promote renal phosphate wasting by impairing the expression of type II cotransporters and by reducing the production of vitamin D_3 and increasing its metabolism. FGF-23 both reduces the expression of the 1 alpha(OH)ase enzyme reducing the production of vitamin D_3 and increases the expression of 24-hydroxylase which metabolizes vitamin D_3 into less biologically active forms. As increasing levels of vitamin D_3 appear to augment FGF-23 concentration, the inhibiting activity of FGF-23 towards vitamin D_3 has been supposed to be part of a negative feed-back mechanism. High levels of FGF-23 have been reported in some diseases like tumor-induced osteomalacia and autosomal dominant hypophosphatemic rickets. The anti-aging hormone klotho has recently been suggested to be a necessary cofactor for FGF-23 action. Other phosphotonins, like secreted frizzled-related protein 4 ($_s$FRP-4), matrix extracellular phosphoglycoprotein and fibroblast growth factor 7, have been shown to reduce renal proximal tubular uptake of phosphate. $_s$FRP-4 seems to act by impairing the expression of type II cotrasporters and by inhibiting vitamin D_3 production, similarly to FGF-23[18]. Total

plasma calcium (tCa) consists of two major forms: protein-bound and unbound part. The protein bound fraction (pCa) is mainly associated to albumin, while the unbound part can be further divided into a freely ionized (iCa) and a portion complexed to anionic species (cCa). Each of these forms is in equilibrium with the others according to different factors like protein level, pH, concentration of citrate, sulphate, biacarbonate, lactate and phosphate[1]. In clinically healthy dogs, iCa, pCa and cCa account for approximately 56%, 34% and 10%. Sick dogs may present disturbances in calcium homeostasis and a tCa concentration within the reference range does not ensure that calcium is distributed among the fractions as would normally be expected. In dogs affected by CKD, in fact, cCa and pCa are significantly different (20.21% and 25.61% respectively) from the concentration reported in clinically healthy population, while iCa shows a similar value (54.32%)[20]. iCa is the biologically active form and the gold standard in the evaluation of the calcemic status. In CKD metabolic acidosis usually develops as a consequence of the low excretion of acid radicals (protons) and phosphate and sulphate compounds through the kidneys, as well as because of low renal absorption of bicarbonate and a decrease in renal ammoniagenesis. In metabolic acidosis iCa can increase in relation to the fact that calcium binds to the same albumin carboxyl group at which H^+ usually binds. Thus, in the presence of acidosis, H^+ radicals do not dissociate easily from the albumin carboxyl group and calcium shows a lower opportunity to bind to albumin. As a result plasma iCa concentration can increase[23]despite of a decrease in pCa fraction. Anyway a recent study reported that only the 24% of CRF dogs in metabolic acidosis showed high concentrations of iCa, while the 56% presented low levels of iCa. The role of metabolic acidosis in predicting reduced concentrations of plasma iCa in CKD dogs was not already documented[23]. Albumin represents the major calcium-binding protein and its concentration can vary significantly in CKD, leading to fluctuations of pCa. The binding of calcium to albumin can also be influenced by plasma pH. Previous studies conducted in human medicine showed that there was no calcium binding to albumin at a pH of 5.5 and that binding steadily increased up to a pH of 8.0 with an almost linear rise through the physiologic pH range[1,21]. In human beings a fall of pH of 1 unit causes approximately a 0.4 mmol/L increase in iCa concentration[5]. By the way no correlation between pH and iCa was observed in CKD dogs[23]. Although alterations in calcium homeostasis are common in critically ill humans and dogs, hypocalcemia often goes unrecognized in intensive care unit (ICU) human patients because it is assumed that tCa

concentration is falsely low due to low level of albumin. Human studies have, in fact, demonstrated that the use of tCa or calcium corrected for albumin cannot be used in an ICU setting to reliably monitor the calcium status. Recent acquisitions in veterinary medicine showed that iCa is the only accurate indicator to assess calcemic status in ICU dogs and that calcium corrected for albumin or total protein leads to overestimation of normocalcemia and underestimation of hypocalcemia[22]. In CKD dogs a discordance of 55% between iCa and tCa was detected, showing that iCa should always be assessed for an accurate evaluation of calcemic status[23]. Although a variety of correcting formulae have been proposed in veterinary and human medicine to permit calculation of the albumin-corrected or iCa from the total calcium and protein concentration, no data support the use of such algorithms in CKD patients[5]. One reason for the poor correlation between adjusted calcium and iCa may be the lack of correction for other molecules able to bind calcium[5]. The amount of cCa in CKD patients can widely vary and in human patients serum cCa is often increased. Calcium, in fact, may form complexes with bicarbonate, citrate, oxalate, citrate and sulphate and high concentrations of these molecules were found in CKD subjects. A recent report on the serum calcium fractions in CKD dogs suggested that the disparity between iCa and tCa was not attributable to differences in pCa or iCa but in cCa[20]. In another veterinary study a slightly significant negative correlation between iCa and phosphate concentration may partially suggest that hyperphosphatemia could contribute to the increase in cCa and decrease in iCa[23]. However a report conducted in 23 dogs with CKD showed that phosphate concentration cannot be used to predict cCa[20]. Serum phosphate concentration increases the cCa concentration and an inverse correlation between iCa and serum phosphate was demonstrated[5]. Available evidences in human beings, cats and dogs showed that calcium and phosphate abnormalities in CKD lead to negative effects on kidney function and survival. In human patients in haemodialysis serum phospate > 6.5 mg/dL increases significantly the risk of mortality. In feline CKD patients, for each 1 mg/dL increase in serum phosphate at time of diagnosis, there is a 11.8% increase in mortality risk. Similarly in dogs hyperphosphatemia has been associated to a rapid progression of CKD and decreased survival[24]. The sequence of events which leads to hyperphosphatemia starts with a reduction in GFR and in renal phosphate excretion. Excess of serum phosphate inhibits the activity of renal 1-alpha hydroxylase, determining a reduction in vitamin D_3 and iCa concentration. As a response

PTH secretion is stimulated. The increase in PTH concentration diminishes tubular phosphate reabsorption, increasing phosphaturia. Initially, this mechanism restores vitamin D_3 and iCa concentration but at expenses of a still high activity of PTH. Every time PTH returns to its initial concentration further phosphate retention occurs. On the contrary, each time GFR decreases the mechanism restarts preserving phosphate balance. Once maximal inhibition of tubular phosphate reabsorption is exceeded and GFR continues to decrease, vitamin D_3 remains low despite of a persistently high PTH concentration and severe hyperphosphatemia. In human beings phosphate retention starts at the early stages of CKD. In these patients, in fact, serum PTH begins to increase when serum phosphate concentration is still within the reference range. Available data suggest that hyperphosphatemia is a common finding even in CKD canine patients, showing a prevalence between 44.2% and 94.9%. A recent study reported that SHPT represents the most common abnormality of calcium-phosphate metabolism, affecting the 75.9% of CKD dogs and that this value increases with the severity of the disease. The prevalence of SHPT was > 96% in stage 3 and 4 and serum elevation of PTH can anticipate the onset of hyperphosphatemia. This finding is in agreement with a study performed in cats where the prevalence of SHPT was about 84%. Anyway some dogs can present phosphate retention at early stages of CKD as it occurs in humans[24].
Vascular calcification in CKD can be determined by different mechanisms such as passive precipitation of calcium phosphate due to high extracellular concentrations, increased activity of osteogenic inducers and deficiency of calcification inhibitors[10]. C x P product represents a clinically relevant tool to estimate cardiovascular risk in CKD and it can be accurately assessed through total or ionized calcium. High serum Ca x P product levels are responsible for a major risk of ectopic calcifications that can contribute to soft-tissue calcium phosphate deposits. Chronic kidney disease-mineral and bone disorder (CKD-MBD) defines a triad of interrelated abnormalities of serum biochemistry, bone and vasculature often associated with CKD[17]. In human medicine the relative risk of death for ESRD patients increases significantly with serum phosphorus higher than 6.5 mg/dL. This increased risk was not diminished by statistical adjustment for coexisting medical conditions, delivered dose of dialysis, nutritional parameters, or markers of noncompliance. The Ca x P product shows a mortality risk trend similar to that seen with serum phosphorus alone. In patients presenting a Ca x P product >72 mg/dL the relative mortality risk was 1.34 relative to those with products of 42 to 52 mg/dL[25].

The new human guidelines for a correct management of the CKD patient underlined the importance of a constant monitoring of serum phosphate and calcium for a more correct management of the CKD patient. Phosphate represents, in fact, a central molecule in the pathogenesis of CKD-MBD and vascular calcification. Frequent monitoring of serum phosphate and treatment of disorders of calcium-phosphate metabolism is essential for reducing the progression of CKD[17].

CHAPTER 8: CARDIOVASCULAR DAMAGE IN CKD: ROLE OF CARDIAC TROPONIN I (CTNI)

Cardiovascular diseases represent the leading cause of morbidity and mortality in ESRD patients, accounting for more than 50% of all deaths[1]. ESRD subjects show the highest risk group for subsequent cardiovascular events with a mortality rate 10-30 times the general population[2]. The importance of cardiovascular disorders in human patients has been demonstrated by the fact that the majority of CKD subjects dies before reaching ESRD. Patients with a reduced GFR show, in fact, a risk of fatal cardiovascular event much higher than the risk of developing ESRD. According to a recent report of the United States Renal Data System cardiac disease accounts for 43% of all-cause mortality in patients submitted to haemodialysis or peritoneal dialysis. Sudden cardiac death associated with arrhythmia or cardiac arrest can reach a 25% of all deaths in ESRD population[4]. CKD patients can be affected by three different forms of cardiovascular disease: atherosclerosis, arteriosclerosis and cardiomiopathy. Atherosclerosis is characterized by focal processes of plaque formation resulting in luminal narrowing and occlusive disease of the vasculature. On the contrary, arteriosclerosis is a non-occlusive remodelling degeneration of the vasculature, characterized by diffuse dilatation and hypertrophy of large arteries, with loss of arterial elasticity and reduced arterial compliance. Arteriosclerosis can determine different clinical manifestations, like left ventricular hypertrophy (LVH), decreased coronary perfusion, high systolic blood pressure and pulse pressure. Cardiomiopathy represents the final step of an adaptation mechanism of the heart towards pressure or volume overload. As heart workload increases, augmented oxygen demand by the hypertrophied left ventricle may exceed its perfusion, leading to ischemia and myocyte death. Initial physiology is often consistent with diastolic dysfunction but, as this process goes on, myocardial fibrosis may develop. The association of myocardial fibrosis and pressure and volume overload may determine the onset of dilated cardiomyopathy. Traditional and non-traditional risk factors of cardiovascular alterations have been identified both in general population and CKD patients[7].

Traditional Risk Factors	Non-traditional Risk Factors
- Older age - Male gender - Hypertension - High LDL - Low HDL - Diabetes - Smoking - Physical inactivity - Menopause - Family history of coronary disease - LVH - White race	***CKD patients*** - Anemia - Volume overload - Abnormal mineral metabolism - Electrolyte imbalance - Albuminuria ***General population*** - Lipoprotein and Apo isoforms and lipoprotein remnants - Homocystein - Oxidative stress/inflammation - Malnutrition - Thrombogenic factors - Sleep disturbances - High sympathetic tone - Altered nitric oxide/endothelin balance

Table 1: traditional and non-traditional risk factors of cardiovascular disease in human CKD patients and in general population. LDL: low-density lipoproteins. HDL: high-density lipoproteins. LVH: left ventricular hypertrophy.

The risk of cardiovascular mortality in ESRD patients has been calculated ≥ 10 to 100 fold higher compared with the age-, sex- and race-matched general population. This category of patients presents, in fact, an increased prevalence of risk factors such as inflammation, high Ca X P product, uremic toxins, anaemia and fluid overload. The pathogenesis of the high cardiovascular injury risk in ESRD patients is multifactor. Anaemia shows a high prevalence in CKD subjects and it is mainly caused by erythropoietin deficiency, erythropoietin hyporesponsiveness and iron deficiency. In patients submitted to haemodialysis, anaemia is frequently associated with eccentric LVH, ventricular dilatation, heart failure and mortality. Mineral metabolism disorders can contribute to vascular calcification and arteriosclerosis,

even though the exact mechanism of action is not completely understood. Conditions of hyperphosphatemia, SHPT, vitamin D_3 deficiency and alterations of calcification promoters and inhibitors are responsible for differentiation of vascular smooth muscle cells into osteoblast-like cells, able to synthesize proteins that increase vascular calcification. Arteriosclerosis, associated with alterations of calcium-phosphate metabolism, can play an important role in stimulating arterial stiffening and vascular and valvular calcifications[3]. Modifications of arterial elasticity have been indicated as predictive factors of obstructive coronary artery disease responsible for myocardial ischemia. Diseased myocardium secondary to coronary ischemia, together with myocardial fibrosis and/or hypertrophy, provides additional substrate for an increased electric instability, which may cause sudden cardiovascular death in uremic subjects. Systolic hypertension and arterial stiffness can determine LVH and represent significant independent predictive risk factors of sudden death[3]. Sodium retention and activation of the renin-angiotensin system are firstly involved in the mechanism of elevation of blood pressure. Human CKD patients, in fact, often show hypertension and elevated plasma concentrations of catecholamines[4]. Anomalies of left ventricular structure and function are very frequent in CKD. Among ESRD patients nearly 15% presents systolic dysfunction, 40% has heart failure and more than 70% LVH. Several studies showed an elevated prevalence of LVH in non-uremic CKD patients. The prevalence of LVH in this category of subjects ranged from 34% to 78%, showing an increasing trend with declining renal function. The wide heterogeneity in the prevalence of LVH in different reports can be attributed to several differences, with regard to the characteristics of the studied population, the method of estimation of GFR, the GFR cut off used to enrol the patients and the exact definition of LVH. Left ventricular wall thickness and diameters generally increase significantly with the progression of renal failure and, in CKD stage 5, a very pronounced left ventricular growth takes place. When left ventricular mass (LVM) is greater than 28% of the value predicted by using an equation including blood pressure, stroke volume, height and gender, LVM is considered not appropriate. Inappropriate left ventricular mass (ILVM) is defined as the value of ventricular mass exceeding the amount needed to adapt to stroke work, according to individual gender and body size and it can be the result of the simultaneous increase of left ventricular diameters and wall thickness. The onset of an ILVM reported a negative impact on cardiovascular prognosis of hypertensive patients. CKD subjects showed a

prevalence of ILVM significantly higher (52.6%) than normal renal function hypertensive patients (30.5%). The prevalence of ILVM appeared to increase with lessening renal function, reaching values of 38% in stage 2, 47.5% in stage 3, 51.35% in stage 4 and 80.7% in stage 5. Although the pathogenesis of LVH is considered to be multifactor, hypertension, alterations of fluid and electrolyte balance and anaemia are the major determinants. LVH represents an adaptive remodelling process, which tries to compensate for an increase in cardiac work, due to volume or/and pressure overload. While hypertension causes concentric hypertrophy, volume overload is responsible for eccentric hypertrophy. Both processes are frequent in CKD patients and a mixed pattern, characterized by increase of both diameters and wall thickness, is often reported. Along with the worsening of CKD, concentric and mixed LVH become more frequent than eccentric hypertrophy. Among hemodynamic factors of LVH, even increased arterial stiffness plays a significant role. Other factors, such as inappropriate activation of renin-angiotensin-aldosterone system, oxidative stress and inflammation have been demonstrated to be involved in the pathogenesis of LVH[5,6]. Increased plasma concentrations of inflammatory markers like CRP, white blood cells and fibrinogen are associated with adverse cardiovascular outcomes in IRIS stage 3 and 4 CKD patients. An independent association between inflammation and risk of cardiovascular disease was found in human patients submitted to haemodialysis. Vitamin E administration was, in fact, related to a lower incidence of cardiovascular problems. At the same time, a reduction in oxidative stress may improve outcomes. Imbalance between pro-oxidant substances and antioxidants leads to increased tissue damage. Inflammation, malnutrition, uremic toxins and dialysis procedures are responsible for stimulating oxidative stress, while plasma protein-associated free thiols reduce oxidant defenses[7]. In advanced renal dysfunction, the reduced clearance of some growth factors has been supposed to take part in the developing of LVH. Carboxy-terminal propeptide of collagen type I (PICP) and cardiotrophyn-1 (CT-1) are involved in the pathogenesis of LVH in hypertensive heart disease. Although PICP seems to depend only on hepatic function and its plasma concentration not to be influenced by renal clearance, a recent study showed higher values of PICP and CT-1 in CKD subjects comparing to normal renal function-hypertensive patients. Moreover, CKD subjects affected by LVH presented significantly higher concentrations of PICP and CT-1 than those without LVH. Sodium/potassium ATPase inhibitors may play a certain role in developing LVH in CKD[5,6].

Very recent acquisitions reported that fibroblast growth factor-23 (FGF-23) shows an independent relationship with LVH (particularly concentric LVH). FGF-23 is a recently discovered hormone secreted by osteoblasts and ostecytes, which helps in maintaining a normal plasma phosphate concentration in CKD. FGF-23 stimulates urinary phosphate excretion and decreases intestinal phosphate absorption by inhibiting vitamin D_3 synthesis. It has been hypothesized that elevated plasma concentrations of FGF-23 in CKD patients are responsible for a non-selective binding of FGF-23 with FGF receptors, normally activated by other factors. This activation could stimulate myocardial fibrosis and contribute to myocardial ischemia, due to a reduction in capillary density and coronary reserve. Increased myocardial fibrosis shows a central role in the pathogenesis of alterations of diastolic function. Recently, a significant correlation between impaired coronary flow and subclinical renal damage has been demonstrated. Left ventricular dysfunction is very frequent among CKD patients and may be associated with the subsequent development of heart failure and mortality. It has been also reported that in ESRD patients diastolic function reduces in parallel with the progression of LVH. However, alterations of diastolic function may occur even in early stages of CKD and in absence of LVH[4]. Early identification of ESRD patients at high risk of cardiovascular disease may improve prognostic accuracy and facilitate more aggressive and focused treatment[1]. Among different candidates, cardiac contractile and regulatory proteins have been demonstrated to be more cardiac-specific and sensitive indicators of myocardial injury. Myofibrillar proteins of striated muscle are expressed as tissue-specific isoforms and these antigens can be differentiated by immunologic methods. Cardiac myosin light chains, cardiac beta-type myosin heavy chains, cardiac alpha-actin, cardiac tropomyosin, as well as cardiac troponin C, are co-expressed in slow-twitch skeletal muscle fibers. Therefore, these proteins cannot be used as specific markers of cardiac injury. Troponins are structural proteins of cardiac and skeletal muscles[9]. Troponin I and Troponin T are present in three different isoforms with an unique structure: one for slow-twitch skeletal muscle, one for fast-twitch skeletal muscle and one for myocardium. The three isoforms are encoded by three different genes[8]. Cardiac Troponin presents two diagnostically relevant forms (cTnI and cTnT), which regulate heart contraction, and it is released into the circulation by injured myocardiocytes within hours. Cardiac Troponin's plasma peak is reached within 2 days and its concentration remains elevated for as long as the injury continues. After acute myocardial injury, cTnI and

cTnT increase in parallel within three to six hour. The early elevation of cTnI and cTnT can be explained by the presence of soluble unbound Troponin pools inside the sarcoplasma, which may be rapidly released after myocardial damage and which account for about 5% amount of cTnI and cTnT in cardiomyocytes. Cardiac Troponins usually peak in parallel, except for patients without reperfusion, in whom cTnI increases about one day and cTnT three to four days after the onset of acute myocardial injury. Although both cardiac Troponins remain increased for at least a four to five day period, cTnT tends to stay elevated longer than cTnI. Reperfusion therapy strongly influences cTnT and cTnI time courses, leading to more rapid increase, higher rate of increase and early peak value[8]. cTnI is not expressed in skeletal muscle during fetal development or in adult skeletal muscle in response to any pathological stimuli. On the contrary, cTnT is expressed in small amounts in skeletal muscle during fetal development but it is not present in adult skeletal muscle. cTnT has been found in regenerating or denervated rat skeletal muscle, probably due to reversion to a fetal-like pattern of expression of Troponin isoforms. This protein is the "gold standard" biomarker of myocardial injury in humans because of its high tissue specificity, diagnostic sensitivity, low basal blood concentration, rapid release and persistence in the blood[10]. Although Troponin is considered a useful marker of myocardial infarction, it is important to underline that elevated plasma concentrations of Troponin reflect a myocardial damage, which may have a different pathogenesis.

Myocardial Infarction	Pathogenesis
- Type 1	- Ischemia due to plaque rupture
- Type 2	- Supply-demand imbalance
- Type 3	- Sudden unexpected cardiac death
- Type 4a	- Percutaneous coronary intervention
- Type 4b	- Stent thrombosis
- Type 5	- Coronary artery bypass grafting

Table 2: human classification of myocardial infarction according to pathogenesis[15]. Type 1 is due to plaque rupture, Type 2 to supply-demand imbalance, Type 3 to sudden unexpected cardiac death, Type 4a to percutaneous coronary intervention, Type 4b to stent thrombosis and Type 5 to coronary artery bypass grafting.

Plaque rupture with superimposed thrombosis within a coronary artery, resulting in myocardial cell death, is the most common mechanism of myocardial infarction in humans. However, plasma Troponin can increase even for conditions of pulmonary embolism, renal failure, sepsis, hypotension and other causes. In patients affected by pulmonary embolism, strain in the right side of the heart and myocardial necrosis are believed to determine Troponin elevation. In sepsis, systemic hypotension causes suboptimal coronary artery blood flow and cytokine release, which are supposed to be responsible for Troponin leakage from myocytes[15]. Among CKD humans, cardiac Troponin is often elevated in the absence of acute coronary syndrome and cTnT is more frequently increased than cTnI in asymptomatic ESRD patients. High cTnT concentration shows, in fact, a prevalence between 30% and 85%, towards a value between 5% and 18% for cTnI. Although both cardiac Troponins tend to increase with progression of CKD, cTnT was strongly predictive of poor short prognosis regardless of creatinine clearance. cTnT seems to have greater prognostic importance among patients affected by mild to moderate degrees of CKD. Anyway, the lower incidence of cTnI elevation and lack of expression in non-cardiac tissue, led to the suggestion that cTnI may be a more specific diagnostic and prognostic marker of myocardial injury. The increase in cTnT in ESRD patients is very unlikely caused by decreased renal clearance. Both free and bound cTnT are, in fact, relatively large molecules of 37 kD and 77 kD respectively. Moreover, improvement of renal function after kidney transplantation does not seem to reduce pre-existing elevated plasma concentration of cardiac Troponins. High cTnT concentration in asymptomatic ESRD subjects indicates subclinical myocardial necrosis or injury. In uremic cardiac hypertrophy, myocardial capillary growth is unable to keep pace with cardiomyocyte hypertrophy, resulting in increased oxygen diffusion distance and diminished ischemic tolerance of the heart. This condition predisposes patients to subclinical myocardial ischemia and to cellular Troponins leakage. Increased mechanical stress would be responsible for an altered membrane permeability of cardiomyocite, which led to Troponins leakage. Furthermore, the leak of cardiac Troponins from hyperthrophic cardiomyocytes in patients affected by LVH may show the presence of microvascular heart injuries in uremia. Anyway,

the use of cardiac magnetic resonance imaging indicated that high plasma values of cTnT cannot be explained solely by previous subclinical myocardial necrosis of LVH. Additional myocardial alterations, such as fibrosis, may be involved in plasma cTnT elevation[16]. In human patients, even minimally elevated plasma concentrations of cTnT have been related to a significant increase in cardiovascular risk and the higher the elevation of cTnT is, the higher the cardiovascular risk[1] is.

Recently veterinary publications[12,13,17] have reported increased cTnI concentrations in dogs affected by different cardiac congenital and acquired diseases, including arrhythmogenic right ventricular cardiomyopathy in Boxer dogs, pericardial effusion, cardiac contusion, experimental infarction, cardiac injury caused by pacing, positive inotropic and cardiotoxic drug administration and neoplasia[11]. cTnI is part of the Troponin complex (Troponin I, C and T) within the sarcomere in myocardial cells and it is important for excitation-contraction coupling. The majority of cTnI is bound within the thin filaments of the contractile apparatus, whereas a small part of the protein remains free in the cytosol. In case of myocardial injury, cTnI is released into the blood stream and its concentration starts to rise within two hours, with a peak after 12-24 hours. A persistent increase in cTnI plasma concentration is suggestive of irreversible and active cardiomyocite damage. Furthermore, in dog models of acute coronary occlusion cTnI concentration correlates with infarct size, suggesting a potential role of cTnI as a marker for the extent of myocardial injury. Canine patients with acquired cardiac diseases and high plasma values of cTnI reported shorter survival times. Long survival times were associated with low cTnI concentrations, which indicate absence of significant active cardiomyocyte damage. Dogs affected by cardiac diseases with different surviving times showed different cTnI concentrations. Dogs surviving more than 1 year, 2 years and 3 years presented median cTnI levels of 0.18 ng/mL, 0.07 ng/mL and 0.05 ng/mL respectively[11]. Elevated plasma values of cTnI are associated with many cardiac diseases like congestive heart failure, cardiomyopathy, ventricular arrhytmias, pericardial effusion, valvular disease, sub-aortic stenosis, experimentally-induced infarction, third-degree heart block, endocarditis and white muscle disease[10]. A particular correlation between high cTnI and mixomatous mitral valve disease (MMVD) has been found in dogs. Plasma cTnI concentration increases with increasing severity of MMVD. Cardiac release of cTnI starts in the early stages of MMVD. Because the half-life of cTnI is < 70 minutes, the increased cTnI

concentration is mainly due to an ongoing release of cTnI, caused by a continuous remodelling process, rather than an acute mechanism[14]. A significant increase in cTnI has been reported even in pathologic conditions other than cardiac diseases, like severe respiratory diseases, pancreatitis, moderate-to-marked anaemia, Addison's and Cushing's disease, advanced renal failure and neoplasia. Although the underlying cause of myocardial injury in these conditions remains unclear, ischemia and hypercatecholaemia seemed to be involved.

Primary cardiac disease/injury	**Infectious disease**
- congestive heart failure - cardiomyopathy - ventricular arrhythmia - pericardial effusion - valvular disease - sub-aortic stenosis - experimentally-induced cardiac infarction - third-degree heart block - endocarditis - white muscle disease - cardiac pacing - cardiac surgery	- babesiosis - ehrlichiosis - leptospirosis - foot and mouth disease - sepsis - endotoxaemia
Toxicity	**Miscellaneous**
- Adrenergics (phenylpropanolamine, isoproterenol) - Anthraquinones (doxorubicin, daunorubicin) - Monensin - Fumonisisn B1 - Viper envenomation	- Trauma - Gastric dilatation/volvulus - Severe respiratory disease - Heat stroke - Severe exertion - Renal failure - Anaemia - Ageing - Pancreatitis - Lymphoma - Visceral neoplasia - Addison's disease - Cushing's disease - Severe colic - Significant weight loss - Downer cow syndrome

Table 3: diseases associated with elevated plasma concentrations of cardiac Troponins in veterinary medicine.

Elevated plasma cTnI concentration is a well-recognized sensitive and specific marker of active cardiac damage but it doesn’t provide any information about the pathogenetic mechanism. Dogs and cats may even present an age-related increase in cTnI concentration, presumably due to a sub-clinical age-associated deterioration of cardiac function. This dysfunction is more common in older dogs than in cats and no association between cTnI concentration and breed has been reported. Anyway transient release of cTnI can occur after reversible cardiac injury, caused by intense exercise[11]. Although approximately 78% of dogs and 72% of cats affected by CKD presented high plasma levels of cTnI, the exact pathogenesis is still unknown. Despite cTnI being eliminated by the kidney, the severity of CKD does not correlate with the cTnI concentration. The possible causes of the elevation of cardiac Troponins in ESRD patients are multiple and often discordant, according to different studies. Among different hypothesis, uremic myopathy, expression of fetal cardiac Troponins in skeletal muscle, altered protein clearance, abnormal protein metabolism, silent myocardial injury, microinfarctions, LVH and uremic toxins have been taken into consideration[9]. In human medicine the most reliable hypothesis reports that high plasma levels of cTnI in CKD patients are caused by a concomitant coronary artery disease. As coronary artery disease is not a feature in veterinary medicine, the relationship between CKD and elevated cTnI concentrations should be further investigated[17].

CHAPTER 9: ACUTE-PHASE INFLAMMATION AND CKD PROGRESSION: ROLE OF C-REACTIVE PROTEIN (CRP)

Acute phase proteins (APPs) enclose a group of proteins the plasma concentration of which changes rapidly in response to the action of different stressors. APPs are divided into positive and negative proteins according to an increase or decrease of their plasma levels during inflammation. C-reactive protein (CRP), haptoglobin (Hp), ceruloplasmin (Cp), serum amyloid A (SAA), alpha-1 acid glycoprotein (AGP) and fibrinogen are considered positive APPs, while albumin and transferrin negative ones[1]. The majority of APPs is produced by the liver, through the stimulating activity of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α. These cytokines are secreted by activated monocytes in response to local tissue injury or bacterial toxins. Once secreted, pro-inflammatory cytokines diffuse into the blood stream, where they act both locally, at the site of inflammation, and systemically towards target organs. Their serum concentration increases within few hours after the starting stimulus and decreases progressively[2]. Pro-inflammatory cytokines are divided into two groups (IL-1 type cytokines and IL-6 type cytokines), which interact with different kinds of receptors of hepatocytes. IL-1 type cytokines stimulate different cellular types to release IL-6 cytokines, showing a pro-inflammatory action. On the contrary, IL-6 type cytokines seem to act a negative feed-back towards the production of IL-1 type cytokines. Il-1 type and IL-6 type cytokines show both common and specific functions.

IL-1	IL-6	TNF-α
- Activation of stroma, condrocytes and epithelium in response to localised tissue damage - Regulation of B-lymphopoiesis in bone marrow - Mediation of tissue	- Induction and differentiation of cytotoxic T lymphocytes - Stimulation of differentiation of hematopoietic stem cells - Modulation of production of IL-1	- Induction of IL-1 production - Participation in cell destruction by suppressing protein synthesis - Elicit local endothelial injury

infiltration of leukocytes through IL-8 - Osteoblast activation and bone and cartilage degradation	and TNF	
- Induction of hepatic acute phase response - Fever induction - Activation of T-, B- and NK cells - Induction of IL-2 in T-cells		

Table 4: main specific and common functions of IL-1, IL-6 and TNF-α[2].

In dogs and cats, APPs can be classified in major, moderate or negative, according to the modification in their plasma concentration when an inflammatory stimulus occurs. Major APPs show a plasma concentration ten to one hundred times the physiologic range, while moderate APPs show a plasma concentration twice to ten times. Negative APPs, instead, are characterized by a plasma concentration below the physiological range. In dogs, CRP and SAA are considered major APPs, while Cp, Hg and AGP are considered moderate ones. In cats, major APPs enrol AGP and SAA, while Hp is classified as moderate acute phase protein. In both canine and feline patients albumin is considered a negative acute phase protein[2]. The response pattern of APPs is, in fact, specie specific, with the exception of albumin, which plasma concentration reduces between 10% and 30% in all mammalians[3]. Generally, SAA and CRP show a higher increase in plasma concentration than Hg. In physiologic conditions, the basal plasma concentrations of SAA and CRP are undetectable, so the relative increase in plasma concentration during acute inflammation is significantly more elevated. However, SAA and CRP plasma concentrations are substantially lower than other APPs with negligible normal plasma concentrations[2]. Acute phase response is part of a general defence response towards tissue injuries, which involves different APPs. A number of APPs is, in fact, directly involved in the protection of the host[4] and its functions well understood. Hp, for example, is

able to bind hemoglobin released by damaged erythrocytes and, together with hemopexin and transferrin, both to alleviate the detrimental effects of free iron and to reduce the availability of free iron for invading bacteria. Although the biological function of other APPs has not been completely established yet, many APPs showed to be involved in a down-regulation process of pro-inflammatory cytokines production and activity in monocytic cells. Due to a relatively short half-life in serum and high response in diseased animals, APPs can be considered a valid measure of a systemic response to an initiating stimulus at the time of blood sampling. The maximum serum concentration of APPs is typically reached within 24 and 48 hours. If no further stimulus occurs, a decline in serum concentration and feed-back regulation, leading to resolution of inflammation, are seen within four to seven days from the starting process.
CRP is formed by five no-covalently associated polypeptide subunits, with a total molecular size of approximately 115 kDa[5]. This protein has been discovered in blood of human patients in acute phase of pneumococcal pneumonia and its name derives by the ability of CRP to bind pneumococcal C-polysaccharide[2]. The blood concentration of CRP is known to be elevated from one hundred to one thousand times within 24 and 48 hours after inflammatory irritation and it is widely used in human medicine as a sensitive marker of inflammation. Similarly in veterinary medicine, CRP shows high in various inflammatory conditions, such as infections[6], surgical trauma[7], neoplasia[8,9], pyometra[10], acute pancreatitis[11], immune-mediated haemolytic anaemia, arthritis, glomerulonephritis and experimental inflammation[1]. In human medicine both traditional and non-traditional risk factors of cardiovascular disease in CKD have been identified. Traditional cardiovascular risk factors include advanced age, gender, hypertension, diabetes mellitus, smoking, hyperlipidemia, left ventricular hypertrophy, heart failure, physical inactivity and are indicated into the Framingham risk equations. Although the high prevalence of traditional risk factors, the severity and extend of cardiovascular complications in CKD patients underline the role of non-traditional factors. These factors are not included into the Framingham equations and enrol albuminuria, anaemia, disorders of bone and mineral metabolism, inflammation and oxidative stress[13,14].

Traditional risk factors	Non-traditional risk factors
- older age - male gender - hypertension - high LDL - low HDL - diabetes mellitus - smoking - physical inactivity - menopause - family history of CVD - LVH - Heart failure	- albuminuria - anaemia - homocysteine - inflammation - oxidative stress - disorders of mineral metabolism - vascular calcification - uremic bone disease - malnutrition/protein wasting - sympathetic activation - extracellular fluid volume overload - coagulation disorders - altered nitric oxide/endothelin balance - insulin resistance - subclinical hypothyroidism - uremic toxins - adipokine imbalance

Table 5: traditional and non-traditional risk factors of cardiovascular disease in human CKD patients[12].

Chronic inflammation has been widely described in people affected by CKD since the early stages of the disease and its prevalence increases with the worsening of renal failure. Together with oxidative stress and endothelial dysfunction, inflammation represents one of the most important contributors of the development and progression of atherosclerosis, which is considered the link between CKD and cardiovascular diseases. The inflammatory mechanism occurring in CKD patients has a multifactor origin. Even in the absence of overt clinical illness, underling diseases may cause acute phase inflammation and predispose patients to infections. The prevalence of inflammation tends to increase along with the progression of CKD, especially in ESRD patients in which uraemia and impaired immune response amplify the inflammatory condition. Uraemia can play a fundamental role in stimulating inflammation. Uremic toxins are, in fact, directly responsible for releasing cytokines. Guanidines were shown to enhance the production of TNF-α by monocytes and advanced glycation-end products (AGE-s) have been reported to increase cytokine release. Furthermore the reduction in renal clearance determines an increase in plasma concentration of pro- and anti-inflammatory cytokines and other mediators, such as AGE-s. Although it has been

questioned which was the overall effect of pro- and anti-inflammatory cytokines retention, the net result appears to be an accumulation of pro-inflammatory mediators. Overhydrated patients show elevated plasma levels of several cytokines and endotoxins comparing to stable, edema-free people with chronic heart failure. Diuretic treatments, in fact, reported significant decreases in plasma endotoxin concentrations. Moreover, the negative correlation between serum albumin and excessive extracellular fluid volume suggested that overhydration may lead to reduced albumin synthesis by increasing endotoxin and cytokine levels. Up-regulation of inflammatory cytokines has also been shown in patients affected by heart failure. Myocardial cells are, in fact, able to produce TNF-α in response to increased left ventricular pressure and volume overload. In heart failure, myocardial expression of TNF-α and IL-6 can also increase due to a chronic β-adrenergic stimulation, determined by an elevated symphathetic activity. In haemodialysis patients CRP levels correlate positively with left ventricular mass and negatively with ejection fraction. In this class of patients, several studies reported a strong correlation between LVH and inflammatory state. Oxidative stress seems to be involved in stimulating inflammation and extensive investigations demonstrated a bidirectional and synergistic connection between oxidative stress and inflammatory process[12]. A strict relationship between extra-osseous calcifications and inflammatory syndrome has been widely demonstrated, showing a significant increase in cardiovascular mortality rate[15]. Chronic inflammatory response precedes the formation of atherosclerotic plaques and involves cytokines and other mediators, which can regulate the osteogenic differentiation of vascular cells[16]. Moreover, valvular and perivalvular calcifications of the mitral annulus and aortic valve can be easily complicated by infective endocarditis.

CHAPTER 10: OXIDATIVE STRESS AND CKD PROGRESSION: ROLE OF α-TOCHOPHEROL

In human medicine it has been widely demonstrated that cardiovascular diseases represent the major cause of morbidity and mortality in CKD patients. In fact, as GFR reduces the prevalence and severity of cardiovascular injuries increase[1,2]. Cardiovascular alterations seemed to augment not only in ESRD, but also in early stages of the disease. Among patients belonging to IRIS 3 and IRIS 4, the prevalence of cardiovascular diseases is four to five fold higher than observed in general population. In human CKD patients, several cardiovascular risk factors have been recognized. Particularly, a significant importance has been attributed to diabetes, hypertension and obesity. However, cross-sectional studies have demonstrated that traditional cardiovascular disease risk factors are not sufficient to determine the extent of cardiovascular injury risk in CKD and ESRD patients. This finding have led to the conclusion that other risk factors must be involved in the increased prevalence of cardiovascular events in CKD population[3]. Among several factors, oxidative stress was recognized as a powerful risk condition for the development of cardiovascular damage in CKD patients. Oxidative stress can be defined as a state of imbalance between free radicals production and degradation, with a resulting increase and accumulation of free radicals. The more common forms of free radicals are represented by reactive oxygen metabolites (ROS) and reactive nitrogen species (RNS). The majority of ROS production (about 90%) derives from accidental formation in mitochondria during oxygen metabolism. During this process some electrons pass down the electron transport chain and leak away from the main path, going directly to reduce oxygen molecules to superoxide anion[4]. In addition, ROS are deliberately synthesized in phagocytic cells, vascular wall cells and various other tissue, by the action of NAD(P)H oxidase, myeloperoxidase, xanthine oxidase, cycloxygenase and lipoxygenase[5]. ROS involve different compounds like superoxide anion, hydrogen peroxide, hydroxil radical, hypochlorite ion and hydroperoxyl radical, which, at low concentrations, are involved in many physiologic functions. However, when ROS production is excessive, they can react with lipids, carbohydrates, proteins and DNA, altering their structure and function, with subsequent

cellular damages. ROS overproduction and its deleterious effects are controlled by an antioxidant enzymatic and non-enzymatic system. The antioxidant enzymatic fraction is composed by superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase and catalase, while the non-enzymatic one involves gluthathione, thiols, ascorbic acid, α-tochopherol, mixed carotinoids and bioflavonoids. Final result of the antioxidant barrier, of both enzymatic and non-enzymatic form, is to eliminate pro-oxidants and free radicals[3]. In human medicine, although a significant correlation between CKD and oxidative stress has been widely assessed, the origin of this relationship has to be clarified. The most accepted hypothesis derives oxidative stress from a combination of increased ROS production, reduced clearance and dysfunction of antioxidant mechanisms. Alterations of several antioxidant pathways, as erythrocyte SOD, plasma thiol groups, plasma glutathione and glutathione peroxidase, have been reported in CKD patients[3]. It is also to be noticed that total antioxidant capacity of plasma has been demonstrated to be normal or even increased with the progression of renal disease[6]. In CKD numerous biomarkers of oxidative stress have been found above normal range and their increase related to the progression of the disease. GFR, in fact, has been reported to be inversely associated with some products of lipid and protein oxidation. CKD patients are generally characterized by elevated values of lipid oxidation compounds, oxidized low-density lipoproteins (LDL), protein oxidation products, F2 isoprostanes and isolevuglandins and oxidative DNA damage markers[3]. Furthermore, increased activity of NAD(P)H oxidase and subsequent overproduction of superoxide anion in circulating peripheral mononuclear cells, have been reported in IRIS 1 and 2[7]. CKD patients are also affected by concomitant pathologies, like diabetes and hypertension, which can exacerbate oxidative stress. NAD(P)H oxidase, for example, can be activated by hyperglycemia, as well as some cytokines and mechanical stress. Similarly, malondialdehyde and protein carbonylation have shown increased in hypertensive patients with CKD, compared to hypertensive subjects without CKD. Moreover, renin-angiotensin system has reported a significant role in NAD(P)H oxidase activation in vascular smooth muscle cells and kidney. Another potential source of oxidative stress in CKD patients is represented by haemodialysis. This treatment can contribute to oxidative stress in different ways. During haemodialysis session, ROS production can be enhanced by the contact of blood with the dialysis membrane or to the presence of endotoxins in water used for dialysate preparation.

Both conditions are responsible for activation of circulating peripheral mononuclear cells, leading to increasing ROS generation. Furthermore, myeloperoxidase activity has been found augmented during haemodialysis, especially when bioincopatible membranes have been used[8]. Heparin, commonly used as anticoagulant agent during haemodialysis, has also been demonstrated to activate circulating peripheral mononuclear cells. Intravenous administration of heparin can also be responsible for displacement of extracellular-superoxide dismutase from vascular endothelium, by interfering with the binding of the antioxidant enzyme to type C heparin sulphate proteoglycans. Loss of extracellular-superoxide dismutase from vascular wall may contribute to endothelial dysfunction. Finally, haemodialysis can also affect the antioxidant barrier, by reducing plasma concentration of ascorbic acid and α-tochopherol[9].
Oxidative stress has been connected with cardiovascular diseases by the oxidative hypothesis of atherosclerosis. According to this hypothesis the oxidative transformation of LDL would represent the starting event of atherosclerosis and it would contribute to its progression. Oxidized LDL are responsible for adhesion of circulating monocytes to endothelial cells by inducing adhesion molecules (VCAM-1) and specific receptors. Oxidized LDL can also stimulate production and release of monocyte chemotactic protein 1 (MCP-1) from endothelial cells and smooth muscle cells, resulting in increased migration of monocytes into the arterial intima. Furthermore, oxidized LDL show protrombotic activity and increase platelet activation and expression of tissue factor and plasminogen activator inhibitor 1 (PAI-1) on endothelial cells[10,11].

Vitamin E enrols a group of extremely powerful antioxidants composed by four tochopherols (α-tochopherol, β-tochopherol, γ-tochopherol and δ-tochopherol) and four tochotrienols (α-tochotrienol, β-tochotrienol, γ-tochotrienol and δ-tochotrienol), of which α-tochopherol represents the most important and abundant molecule[12]. α-tochopherol is mainly stored in vegetables even if small quantities can be found even in fat of animal origin[13]. After an active intestinal absorption, α-tochopherol seems to be mostly stocked in microsomial membranes, which represent main intracellular pools. The transfer of α-tochopherol from microsomes to mithocondria is mediated by α-tochopherol-transfer proteins (α-TTP). The major affinity of α-TTP for α-tochopherol, compared to other isomers, may explain why α-tochopherol is the most represented form of Vitamin E in the body, despite the higher concentration of γ-

tochopherol in food[14]. In physiologic conditions α-tochopherol absorption depends on the quantity of dietary fats introduced and ranges within 30% and 80%, with a high inter-individual variability. This difference may be explained by inter-individual variability in the expression and activity of Vitamin E intestinal receptors[15]. α-tochopherol is metabolized by the liver and it is transported in plasma by lipoproteins[16]. Its tissue distribution does not appear uniform and different organs show different concentration of α-tochopherol. Highest concentrations are reached in fat tissue and surrenal glands, while lowest ones are present in red blood cells and kidney[17].

Tissue	α-tochopherol (μg/g tissue)
Plasma	9.5
Red blood cells	2.3
Platelets	30
Fat	150
Kidney	7
Liver	13
Muscle	19
Ovary	11
Testis	40
Uterus	9
Heart	20
Surrenal gland	132
Pituitary gland	40

Tabella 6: α-tochopherol concentration expressed as μg/g of tissue in different organs and tissues

α-tochopherol shows different biological antioxidant and not antioxidant functions. As an antioxidant compound, α-tochopherol acts as a chain-breaking molecule towards lipid

oxidation, through a rapid and non-enzyme mediated action. During lipid oxidation process, in fact, α-tochopherol is able to bind peroxide radicals, avoiding their binding to lipid targets. Beside antioxidant action, α-tochopherol is responsible for several functions such as inhibiting the phosphorilation of protein C kinase, platelet aggregation and endothelial adhesion of monocytes. Recently a modulating gene activity has been discovered[18,19]. α-tochopherol is considered the most powerful fat-soluble antioxidant known in nature and it works through the inhibition of the oxidation of low density lipoproteins (LDL) by oxidants[20]. Several studies have reported that the protein fraction of LDL (apolipoprotein B_{100}) may be carbamylated at lysine or the terminal protein amino acids. Carbamylation is the result of a non-enzymatic post-translational modification of a protein by urea-derived cyanate, normally elevated in plasma of human CKD patients. It has also demonstrated that carbamylation of LDL represents a significant non-traditional risk factor for the onset of cardiovascular diseases. According to oxidative hypothesis of atherosclerosis, atherosclerotic plaques would be originated by the cooperation of foam cells and macrophages, covered by a fibrous cap. Injured endothelial cells, in fact, attract monocytes, which have previously ingested carbamylated LDL, originating foam cells[21]. LDL, in their native state, are not atherogenic and they must undergo oxidative modifications before becoming atherogenetic[3]. Several studies have reported a significant increase in carbamylated LDL concentration in uremic patients and the elevation in oxidized LDL has been shown to be related with the severity of the disease. Strong and independent relationship between oxidized LDL and cardiovascular alterations has been reported in CKD population, as well as a large number of evidences have suggested oxidized LDL as leading cause of atherosclerosis[10]. Increase in plasma concentrations of carbamylated LDL has been associated with concomitant alteration in antioxidant defence[22]. Anyway, the role of serum α-tochopherol reduction in increasing cardiovascular risk in CKD patients is still controversial. In human CKD patients, serum levels of α-tochopherol are generally reduced in long-term haemodialysis, as long as other nutritional parameters and lipid profile. Many studies regarding α-tochopherol in CKD have reported that α-tochopherol alone cannot be considered a mortality risk. Furthermore, α-tochopherol has not been able to decrease mortality and, at high doses, mortality can even increase[23,24]. Prolonged oral α-tochopherol administration in haemodialysis patients has seemed to decrease the activity of the antioxidant enzyme superoxide dismutase and total

plasma antioxidant system, showing a pro-oxidant effect[25]. In other studies, instead, α-tochopherol supplementation has been associated with beneficial effects on heart diseases and on lipid peroxidation. In haemodialysis subjects, oxidized LDL concentration has been reported to be significantly decreased after a 16 week α-tochopherol integration[26]. In mice, high concentrations of α-tochopherol supplementation have been demonstrated to significantly reduce cisplatin-induced nephrotoxicity, restoring normal levels of the renal antioxidant barrier. This effect has seemed to be dose related. Dietary α-tochopherol supplementation at the dosage of 500 mg/kg of body weight has shown more effective than 250 mg/kg in improving antioxidant barrier[27]. Moreover, dietary integration of α-tochopherol (200 IU/kg) has been capable to reverse acquired glomerulosclerosis and tubulointerstitial injury in rat models with CKD[28]. In veterinary medicine, no studies regarding serum levels of α-tochopherol in CKD are present. The only study in feline CKD patients, has reported only a non-significant tendency to increased values of oxidative status, compared to control group. In these patients, the combined administration of α-tochopherol, vitamin C and β-carotene has shown a protective action towards oxidative injuries of DNA[29]. Further investigations referring to α-tochopherol concentration in CKD animals and its relationship with the severity and the progression of the disease are strongly needed.

CHAPTER 11: MATERIALS AND METHODS

Treatment of animals

The research project involved a total of 301 privately owned dogs of different breed, gender, age and weight, at different stages of CKD, referred to the Veterinary Teaching Hospital "Mario Modenato" between December 2008 and December 2010 for nephrological consultation. 291 patients showed a diagnosis of CKD reported by referring veterinarian, while 10 subjects were referred for a GFR evaluation. A total of 125 clinically healthy (CH) dogs of different breed, gender, age and weight, presented to the Veterinary Teaching Hospital "Mario Modenato" between December 2008 and December 2010 for routine annual check-up, was involved as control group, after owners' informed consent. Each patient was submitted to historical record, clinical evaluation and blood sample (5 ml of volume) for assessing the standard biochemistry panel used to diagnose CKD. Renal panel involved plasma creatinine and urea, serum albumin, total proteins, total calcium, phosphate and ionized calcium, together with complete blood count (CBC) and complete urinalysis. After a 12 hour fasting period, all dogs (both CKD and clinically healthy) were submitted to blood sampling (5 ml of volume) and blood was collected both in heparinised tubes (for obtaining plasma) and in tubes without anticoagulant agents (for obtaining serum). 10 dogs with plasma creatinine within the normal range were submitted to GFR test through the plasma clearance of iohexol. CKD patients were classified according to the International Renal Interest Society guidelines in IRIS 1, IRIS 2, IRIS 3 and IRIS 4. For each patient, Ca X P product (Ca X P), calcium corrected for albumin (tCaAlb) and calcium corrected for total proteins (tCaPt) were calculated. Ca X P was assessed by multiplying serum total calcium per phosphate. tCaAlb was determined through the following formula: tCaAlb (mg/dl) = total calcium (mg/dl) – albumin (g/dl) + 3.5. tCaPt was determined through the following formula: tCaPt (mg/dl) = total calcium (mg/dl) – (0.4 x total proteins {g/dl}) + 3.3.All dogs' owners were asked for informed consent, in order to store serum samples for research purposes. 44 of 301 owners of CKD patients and 20 of 125 owners of CH dogs accepted to be enrolled in the present research project. IRIS 1 group was composed by 13 dogs, IRIS 2 group by 7 dogs, IRIS 3

group by 13 dogs and IRIS 4 group by 11 dogs. CH group was composed by 20 dogs. Serum of each patient was divided into four parts for the determination of total calcium X phosphate product (Ca X P), cardiac troponin I (cTnI), C reactive protein (CRP) and α-tocopherol. Control group was composed by 125 subjects for total calcium, ionized calcium, tCaAlb and tCaPt and by 20 subjects for Ca X P, cTnI, CRP and α-tocopherol.

Plasma creatinine

Plasma creatinine was determined at the Laboratory of Clinical Haematology and Biochemistry of the Veterinary Teaching Hospital "Mario Modenato" through a commercial kit based on a cinetic method, which uses alkaline picrate in the Jaffe reaction. Analysis was performed by *SLIM*® analyzer (SEAC, Firenze, Italia).

Plasma urea

Plasma urea was determined at the Laboratory of Clinical Haematology and Biochemistry of the Veterinary Teaching Hospital "Mario Modenato" through a commercial kit based on a cinetic UV method. Urease catalyzes the hydrolysis of urea into carbon dioxide and ammonia, which, at the presence of glutamate dehydrogenase, react with ketoglutarate sodium and NADH generating NAD and causing a reduction in absorbance. Analysis was performed by *SLIM*® analyzer (SEAC, Firenze, Italia).

Serum albumin

Serum albumin was determined at the Laboratory of Clinical Haematology and Biochemistry of the Veterinary Teaching Hospital "Mario Modenato" through a commercial colorimetric kit based on the bromocresol green (BCG) reaction. Analysis was performed by *SLIM*® analyzer (SEAC, Firenze, Italia).

Serum total proteins

Serum total proteins were determined at the Laboratory of Clinical Haematology and Biochemistry of the Veterinary Teaching Hospital "Mario Modenato" through a commercial colorimetric kit based on Biuret reaction. In alkaline conditions, proteins link to copper anions

forming blue-violet complexes, while peptides link to copper anions forming red complexes. Colour intensity is proportional to the concentration of proteins in the sample. Analysis was performed by *SLIM*® analyzer (SEAC, Firenze, Italia).

Serum total calcium

Total calcium (tCa) was determined at the Laboratory of Clinical Haematology and Biochemistry of the Veterinary Teaching Hospital "Mario Modenato" through a colorimetric method, which uses a Cresolphthalein Complexone (CPC) diagnostic kit. Analysis was performed by *SLIM*® analyzer (SEAC, Firenze, Italia).

Serum phosphate

Phosphate was determined at the Laboratory of Clinical Haematology and Biochemistry of the Veterinary Teaching Hospital "Mario Modenato" through a direct UV kit based on Molybdate reaction. In acid conditions, inorganic phosphate reacts with ammonium molybdate forming a coloured complex. Colour intensity is proportional to the concentration of phosphate in the sample. Analysis was performed by *SLIM*® analyzer (SEAC, Firenze, Italia). Ca X P was calculated by multiplying total calcium per phosphate.

Serum ionized calcium

Ionized calcium was determined at the Laboratory of Clinical Haematology and Biochemistry of the Veterinary Teaching Hospital "Mario Modenato" through a selective ion hemogasanalysis machine STAT PROFILE® pHOx Plus (GEPA, Milano, Italy), which measures potential difference of the sample.

Serum cardiac troponin I (cTnI)

cTnI was assessed by an immunometric method. Blood was collected into tubes without anticoagulant and centrifuged at 3000 rpm for 3 minutes to obtain serum. Serum was stored at -20°C till analysis. Samples were sent to *Vet Med Labor GmbH, Division of IDEXX Laboratories* – Mörikestraße 28/3, 71636 Ludwigsburg, Deutschland for the determination of cTnI. Serum cTnI was determined through immunometric method (IMMUNOLITE 2000®

Immunoassay System) using enzyme-amplified chemiluminescence technique. This method was based on a solid phase, covered by murine monoclonal anti-troponin I antibodies, and on a liquid phase, composed by bovine alkaline-phosphatase linked to goat polyclonal anti-troponin I antibodies. Serum samples (200 μl) were incubated together with reagent and solid phase for 30 minutes. Then samples were washed and centrifuged till the chemiluminescent substrate was added. As much intense luminescence was as much enzyme was linked. A value of serum cTnI > 0.20 ng/ml was considered pathologic.

Serum C-reactive protein (CRP)

CRP was assessed by an immunoturbidimetric method. Blood was collected into tubes without anticoagulant and centrifuged at 3000 rpm for 3 minutes to obtain serum. Serum was stored at -20°C till analysis. Samples were sent to *Vet Med Labor GmbH, Division of IDEXX Laboratories* – Mörikestraße 28/3, 71636 Ludwigsburg, Deutschland for the determination of CRP. Serum CRP was determined through a RANDOX immunoturbidimetric kit® for CRP (Vet Med Lab, IDEXX, Germany). 100 μl of serum were added to assay buffer, which was composed by polyethylene glycol (at a maximum of 4%), tris/HCL buffer (20 mmol/l at pH 7.4) and sodium chloride (150 mmol/l). Then samples were added to antibody reagent, which was composed by human anti-CRP antibodies.

Serum α-tochopherol

α-tochopherol was assessed through a HPLC system by using a Chromosystems-Diagnostic Kit HPLC & LC/MS® (Munchen, Germany). 200 μl of serum were added to 20 μl of internal standard and to 25 μl of precipitation reagent I. Samples were vortex for 30 seconds and added with 400 μl of precipitation reagent II. Then samples were vortex for 30 seconds and centrifuged at 10000 rpm for 10 minutes. 50 μl of supernatant were submitted to HPLC analysis. The HPLC system consisted of a Series 200 Perkin Elmer gradient Pump coupled to a Series 200 Perkin Elmer variable UV detector, which was set at 325 nm and 254 nm. HPLC was interfaced with a personal computer by using an interface SERIES 600 Perkin Elmer (Norwalk, CT, USA). Integration of pikes was performed through Turbochrome Navigator software (Perkin Elmer). A chromatographic column (CHROMOSYSTEMS®, Munchen,

Germany) was used. Instruments showed a wave length of 325 nm, which was switched to 295 nm after 3.5 minutes. The flux was set at 1.5 ml/min and temperature of the column was 25°C.

Statistical analysis

For all 301 patients, belonging to different IRIS stages (IRIS 1, IRIS 2, IRIS 3 and IRIS 4), mean values of plasma creatinine and urea, serum albumin, total proteins, total calcium, phosphate, ionized calcium, tCaAlb and tCaPt were calculated. Pearson correlation analysis ($p<0.05$) between ionized calcium and total calcium, ionized calcium and tCaAlb, ionized calcium and tCaPt was performed in all IRIS stages. For control group subjects (CH), mean values of plasma creatinine and urea, serum albumin, total proteins, total calcium, phosphate, ionized calcium, tCaAlb and tCaPt were calculated. Correlation analysis ($p<0.05$) between ionized calcium and total calcium, ionized calcium and tCaAlb, ionized calcium and tCaPt was performed through Pearson test.

Average Ca X P, cTnI, CRP and α-tochopherol were calculated for both CKD patients with parameters above and below the reference range and for control group.

Variance analysis ($p<0.05$) among mean serum values of Ca X P of control group and IRIS 1, 2, 3 and 4 was determined through ANOVA test. If significant difference among control group and IRIS stages was present ($p<0.05$), Dunnett's and Tukey's post-tests were performed. Pearson correlation test ($p<0.05$) between plasma creatinine and Ca X P was assessed for patients belonging to IRIS 1, 2, 3 and 4. Contingency analysis between the number of subjects with Ca X P ≤ 0.7 and > 0.7 was calculated in each IRIS stage through χ^2 test ($p<0.05$). Contingency analysis between the number of patients survived and dead in the group of dogs with Ca X P ≤ and > 0.7 was calculated in each IRIS stage through χ^2 test ($p<0.05$). Survival curve of Kaplan-Meier was determined for patients belonging to the group with Ca X P ≤ and > 0.7.

Variance analysis ($p<0.05$) among mean serum values of cTnI of control group and IRIS 1, 2, 3 and 4 was determined through ANOVA test. If significant difference among control group and IRIS stages was present ($p<0.05$), Dunnett's and Tukey's post-tests were performed. Pearson correlation test ($p<0.05$) between plasma creatinine and cTnI was assessed for patients belonging to IRIS 1, 2, 3 and 4. Contingency analysis between the number of subjects

with cTnI ≤ 0.2 and > 0.2 was calculated in each IRIS stage through χ^2 test ($p<0.05$). Contingency analysis between the number of patients survived and dead in the group of dogs with cTnI ≤ and > 0.2 was calculated in each IRIS stage through χ^2 test ($p<0.05$). Survival curve of Kaplan-Meier was determined for patients belonging to the group with cTnI ≤ and > 0.2.

Variance analysis ($p<0.05$) among mean serum values of CRP of control group and IRIS 1, 2, 3 and 4 was determined through ANOVA test. If significant difference among control group and IRIS stages was present ($p<0.05$), Dunnett's and Tukey's post-tests were performed. Pearson correlation test ($p<0.05$) between plasma creatinine and CRP was assessed for patients belonging to IRIS 1, 2, 3 and 4. Contingency analysis between the number of subjects with CRP ≤ 9.7 and > 9.7 was calculated in each IRIS stage through χ^2 test ($p<0.05$). Contingency analysis between the number of patients survived and dead in the group of dogs with CRP ≤ and > 9.7 was calculated in each IRIS stage through χ^2 test ($p<0.05$). Survival curve of Kaplan-Meier was determined for patients belonging to the group with CRP ≤ and > 9.7.

Variance analysis ($p<0.05$) among mean serum values of α-tochopherol of control group and IRIS 1, 2, 3 and 4 was determined through ANOVA test. If significant difference among control group and IRIS stages was present ($p<0.05$), Dunnett's and Tukey's post-tests were performed. Pearson correlation test ($p<0.05$) between plasma creatinine and α-tochopherol was assessed for patients belonging to IRIS 1, 2, 3 and 4. Contingency analysis between the number of subjects with α-tochopherol ≤ 21.6 ppm and > 21.6 ppm was calculated in each IRIS stage through χ^2 test ($p<0.05$). Contingency analysis between the number of patients survived and dead in the group of dogs with α-tochopherol ≤ and > 21.6 ppm was calculated in each IRIS stage through χ^2 test ($p<0.05$). Survival curve of Kaplan-Meier was determined for patients belonging to the group with α-tochopherol ≤ and > 21.6 ppm. Statistical analysis was performed by using the software GraphPad Prism® 4 for Macintosh, USA.

CHAPTER 12: RESULTS

Ionized calcium, total calcium, calcium corrected for albumin (cCaAlb) and calcium corrected for total proteins (cCaPt)

Ionized calcium, total calcium, calcium corrected for albumin (cCaAlb) and calcium corrected for total proteins (cCaPt) were determined in 301 CKD dogs and in 125 CH subjects. CKD patients showed a mean plasma creatinine of 3.73 ± 3.10 mg/dl. According to plasma creatinine, 22 patients were in IRIS stage 1, 83 in IRIS stage 2, 130 in IRIS stage 3 and 66 in IRIS stage 4. IRIS 1 dogs showed a mean GFR of 43.28 ± 10.92 ml/min/m^2 and an average plasma creatinine of 1.04 ± 0.18 mg/dl. Mean plasma creatinine was 1.68 ± 0.20 mg/dl in IRIS 2, 3.21 ± 0.82 mg/dl in IRIS 3 and 8.41 ± 3.37 mg/dl in IRIS 4. Mean plasma urea was 32.19 ± 10.97 mg/dl in IRIS 1, 66.20 ± 38.22 mg/dl in IRIS 2, 153.93 ± 85.28 mg/dl in IRIS 3 and 284.24 ± 130.04 mg/dl in IRIS 4. Mean serum total calcium was 10.38 ± 0.95 mg/dl in IRIS 1, 9.86 ± 1.44 mg/dl in IRIS 2, 10.20 ± 2.04 mg/dl in IRIS 3 and 9.29 ± 2.06 mg/dl in IRIS 4. Mean serum phosphate was 4.22 ± 1.48 mg/dl in IRIS 1, 4.64 ± 1.77 mg/dl in IRIS 2, 6.59 ± 3.67 mg/dl in IRIS 3 and 15.46 ± 9.13 mg/dl in IRIS 4. Mean serum ionized calcium was 1.28 ± 0.05 mmol/l in IRIS 1, 1.24 ± 0.09 mmol/l in IRIS 2, 1.19 ± 0.20 mmol/l in IRIS 3 and 1.06 ± 0.28 mmol/l in IRIS 4. Mean serum albumin was 2.85 ± 0.59 g/dl in IRIS 1, 3.05 ± 0.74 g/dl in IRIS 2, 2.82 ± 0.79 g/dl in IRIS 3 and 2.74 ± 0.61 g/dl in IRIS 4. Mean serum total proteins were 6.99 ± 0.94 g/dl in IRIS 1, 6.78 ± 1.15 mmol/l in IRIS 2, 6.41 ± 1.26 mmol/l in IRIS 3 and 6.80 ± 1.95 mmol/l in IRIS 4. IRIS 1 patients showed a mean tCaAlb value of 11.02 ± 0.85 mg/dl, IRIS 2 patients a mean value of 10.25 ± 2.05 mg/dl, IRIS 3 patients a mean value of 10.87 ± 1.67 mg/dl and IRIS 4 patients a mean value of 9.98 ± 2.20 mg/dl. IRIS 1 patients showed a mean tCaPt value of 10.96 ± 1.06 mg/dl, IRIS 2 patients a mean value of 10.26 ± 1.44 mg/dl, IRIS 3 patients a mean value of 10.94 ± 1.67 mg/dl and IRIS 4 patients a mean value of 9.67 ± 2.29 mg/dl.

CH subjects showed an average plasma creatinine of 0.90 ± 0.21 mg/dl, a mean plasma urea of 29.31 ± 11.38 mg/dl, a mean serum total calcium of 9.64 ± 0.76 mg/dl, a mean serum phosphate of 3.55 ± 0.77 mg/dl, a mean serum ionized calcium of 1.25 ± 0.06 mmol/l, a mean serum albumin of 3.32 ± 0.47 g/dl, mean serum total proteins of 6.64 ± 0.62 g/dl, a mean tCaAlb of 9.82 ± 0.77 mg/dl and a mean tCaPt of 10.21 ± 0.73 mg/dl.

	CH	IRIS 1	IRIS 2	IRIS 3	IRIS 4
CREAT (mg/dl)	0.90±0.21	1.04±0.18	1.68±0.20	3.21±0.82	8.41±3.37
UREA (mg/dl)	29.31±11.38	32.19±10.97	66.20±38.22	153.93±85.28	284.24±130.04
TCA (mg/dl)	9.64±0.76	10.38±0.95	9.86±1.44	10.20±2.04	9.29±2.06
PHOS (mg/dl)	3.55±0.77	4.22±1.48	4.64±1.77	6.59±3.67	15.46±9.13
ICA (mmol/l)	1.25±0.06	1.28±0.05	1.24±0.09	1.19±0.20	1.06±0.28
ALB (g/dl)	3.32±0.47	2.85±0.59	3.05±0.74	2.82±0.79	2.74±0.61
PT (g/dl)	6.64±0.62	6.99±0.94	6.78±1.15	6.41±1.26	6.80±1.95
TCAALB (mg/dl)	9.82±0.77	11.02±0.85	10.25±2.05	10.87±1.67	9.98±2.20
TCAPT (mg/dl)	10.21±0.73	10.96±1.06	10.26±1.44	10.94±1.67	9.67±2.29

Tabella 2: mean values and standard deviatons of plasma creatinine (mg/dl) and urea (mg/dl), serum total calcium (mg/dl), phosphate (mg/dl), ionized calcium (mmol/l), albumin (g/dl) and total proteins (g/dl) in CH subjects and in CKD patients at different IRIS stages. TCA: total calcium, ICA: ionized calcium, TCAALB: total calcium corrected for albumin, TCAPT: total calcium corrected for total proteins.

One-way ANOVA ($p<0.05$) among mean values of ionized calcium of CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 showed a significant difference ($p<0.0001$, $R^2=0.14$). Tukey's post test showed a significant difference between CH and IRIS 3 ($p<0.05$) and between CH and IRIS 4 ($p<0.001$), between IRIS 1 and IRIS 4 ($p<0.001$) and between IRIS 2 and IRIS 4 ($p<0.001$) and IRIS 3 and IRIS 4 ($p<0.001$). No significant difference was found among CH, IRIS 1 and IRIS 2 and among IRIS 1, IRIS 2 and IRIS 3.

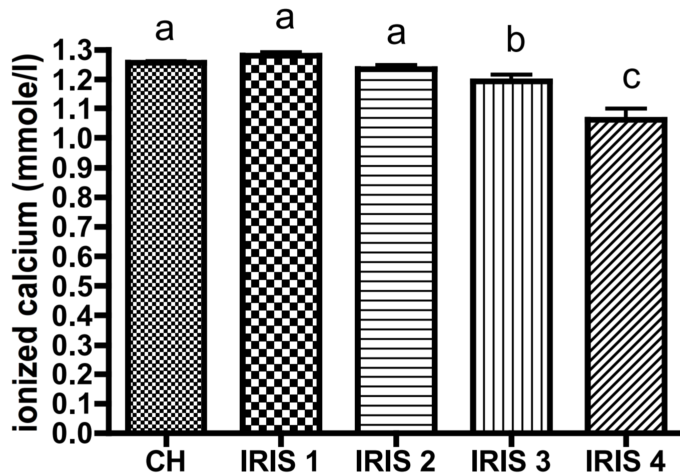

Figura 22: One-way ANOVA test ($p<0.05$) among mean values of ionized calcium in CH subjects and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients. A significant difference was found ($p<0.0001$, $R^2=0.14$). Tukey's post test showed a significant difference among CH, IRIS 1 and IRIS 2 (a) and IRIS 3 (b) and IRIS 4 (c).

ANOVA test	**Ionized calcium**
One-way analysis of variance	
P value	<0.0001
P value summary	***
Are means signif. different? (p<0.05)	Yes
Number of groups	5
F	16,46
R^2	0,1497

Tukey's test	**Mean diff**	**q**	**P value**	**95% Cl of diff**
CH vs IRIS 1	-0,02405	0,9191	>0.05	-0.1264 to 0.078
CH vs IRIS 2	0,01992	1,196	>0.05	-0.04523 to 0.08
CH vs IRIS 3	0,06144	4,074	<0.05	0.002443 to 0.1

CH vs IRIS 4	0,1949	10,72	<0.001	0.1237 to 0.266
IRIS 1 vs IRIS 2	0,04397	1,597	>0.05	-0.06376 to 0.15
IRIS 1 vs IRIS 3	0,08549	3,212	>0.05	-0.01864 to 0.18
IRIS 1 vs IRIS 4	0,2189	7,684	<0.001	0.1075 to 0.330
IRIS 2 vs IRIS 3	0,04152	2,392	>0.05	-0.02639 to 0.10
IRIS 2 vs IRIS 4	0,1750	8,698	<0.001	0.09627 to 0.25
IRIS 3 vs IRIS 4	0,1334	7,085	<0.001	0.05977 to 0.20

Contingency analysis ($p<0.05$), through χ^2 test, among the number of subjects with ionized calcium within, above and below the normal range in CH subjects and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients showed a significant difference ($p<0.0001$). All 125 subjects of CH group and 22 patients of IRIS 1 showed ionized calcium within the normal range. In IRIS 2 group, 70 patients showed ionized calcium within the normal range, while 2 patients were hypercalcemic and 11 hypocalcemic. In IRIS 3 group, 92 patients showed ionized calcium within the normal range, while 5 patients were hypercalcemic and 33 hypocalcemic. In IRIS 4 group, 25 patients showed ionized calcium within the normal range, while 3 patients were hypercalcemic and 38 hypocalcemic.

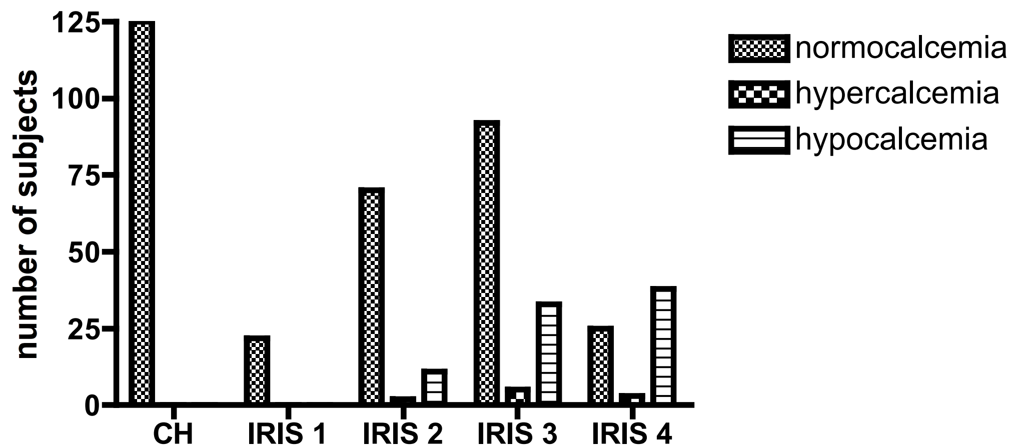

Figura 23: contingency analysis ($p<0.05$) between the number of subject with ionized calcium within, below and above the reference range in CH and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients, showed a significant difference ($p<0.001$)

χ^2 TEST	IONIZED CALCIUM
Chi-square, df	112.7, 8
P value	P<0.0001
P value summary	***
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	Yes

According to the gold standard test (ionized calcium) 22/22 patients of IRIS 1 resulted normocalcemic, while no patients were hyper- or hypocalcemic. 4 patients resulted hypercalcemic and 18 normocalcemic, when total calcium was used. 16 dogs resulted hypercalcemic and 6 normocalcemic, when cCaAlb was used. Finally, 11 patients resulted normocalcemic and 11 hypocalcemic, when cCaPt was used. According to the gold standard test 70/83 patients of IRIS 2 resulted normocalcemic, 2/83 hypercalcemic and 11/83 hypocalcemic. 17 patients resulted hypercalcemic, 61 normocalcemic and 5 hypocalcemic, when total calcium was used. 28 dogs resulted hypercalcemic, 47 normocalcemic and 8 hypocalcemic, when cCaAlb was used. Finally, 23 patients resulted hypercalcemic, 56 normocalcemic and 4 hypocalcemic, when cCaPt was used. According to the gold standard test 92/130 patients of IRIS 3 resulted normocalcemic, 5/130 hypercalcemic and 33/130

hypocalcemic. 41 patients resulted hypercalcemic, 69 normocalcemic and 20 hypocalcemic, when total calcium was used. 28 dogs resulted hypercalcemic, 30 normocalcemic and 9 hypocalcemic, when cCaAlb was used. Finally, 31 patients resulted hypercalcemic, 41 normocalcemic and no one hypocalcemic, when cCaPt was used. According to the gold standard test 25/66 patients of IRIS 4 resulted normocalcemic, 3/66 hypercalcemic and 38/66 hypocalcemic. 9 patients resulted hypercalcemic, 35 normocalcemic and 22 hypocalcemic, when total calcium was used. 8 dogs resulted hypercalcemic, 16 normocalcemic and 6 hypocalcemic, when cCaAlb was used. Finally, 7 patients resulted hypercalcemic, 13 normocalcemic and 7 hypocalcemic, when cCaPt was used.

In IRIS 1 patients, Pearson test ($p<0.05$) between ionized calcium and total calcium showed no significant correlation ($p=0.09$), Pearson test ($p<0.05$) between ionized calcium and cCaAlb showed no significant correlation ($p=0.18$), Pearson test ($p<0.05$) between ionized calcium and cCaPt showed no significant correlation ($p=0.18$). In IRIS 2 patients, Pearson test ($p<0.05$) between ionized calcium and total calcium showed a significant correlation ($p=0.00$), Pearson test ($p<0.05$) between ionized calcium and cCaAlb showed a significant correlation ($p=0.00$), Pearson test ($p<0.05$) between ionized calcium and cCaPt showed a significant correlation ($p=0.00$). In IRIS 3 patients, Pearson test ($p<0.05$) between ionized calcium and total calcium showed a significant correlation ($p=0.00$), Pearson test ($p<0.05$) between ionized calcium and cCaAlb showed a significant correlation ($p=0.00$), Pearson test ($p<0.05$) between ionized calcium and cCaPt showed a significant correlation ($p=0.00$). In IRIS 4 patients, Pearson test ($p<0.05$) between ionized calcium and total calcium showed a significant correlation ($p=0.00$), Pearson test ($p<0.05$) between ionized calcium and cCaAlb showed no significant correlation ($p=0.22$), Pearson test ($p<0.05$) between ionized calcium and cCaPt showed no significant correlation ($p=0.31$).

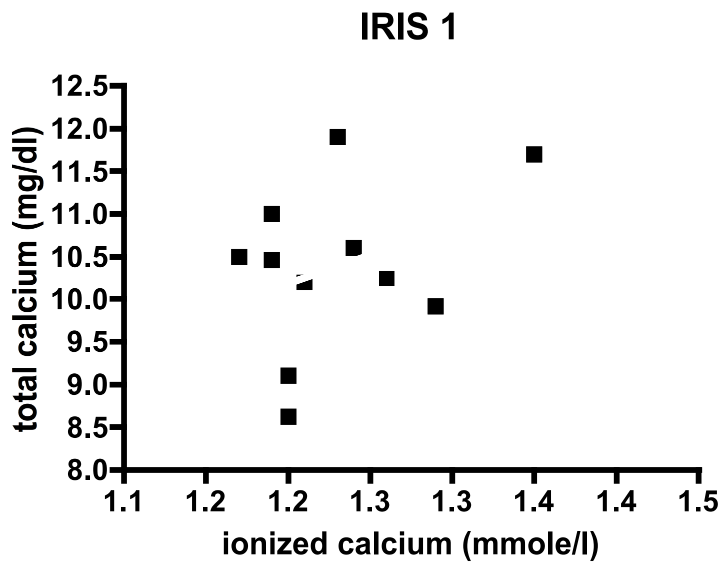

Figure 24: Pearson correlation analysis between ionized calcium (mmole/l) and total calcium (mg/dl) in IRIS 1 dogs. No significant correlation was found. Pearson r: 0.36, P value (two-tailed): 0.09, R^2: 0.13

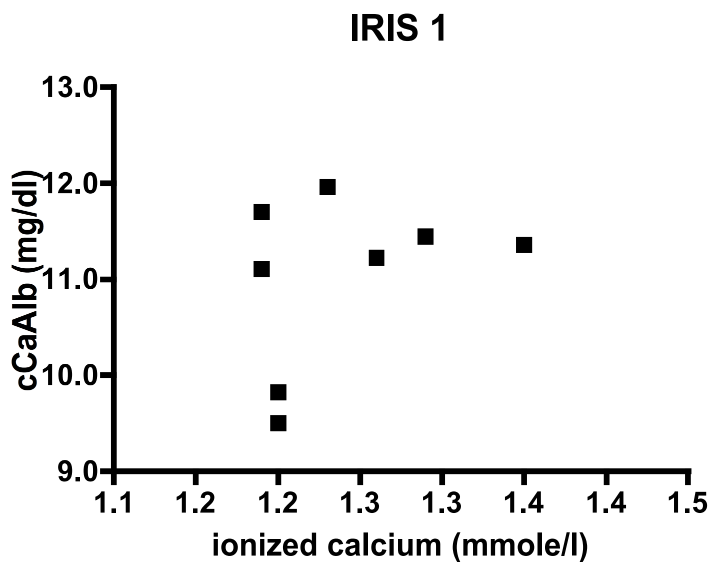

Figura 25: Pearson correlation analysis between ionized calcium (mmole/l) and cCaAlb (mg/dl) in IRIS 1 dogs. No significant correlation was found. Pearson r: 0.34, P value (two-tailed): 0.18, R^2: 0.12

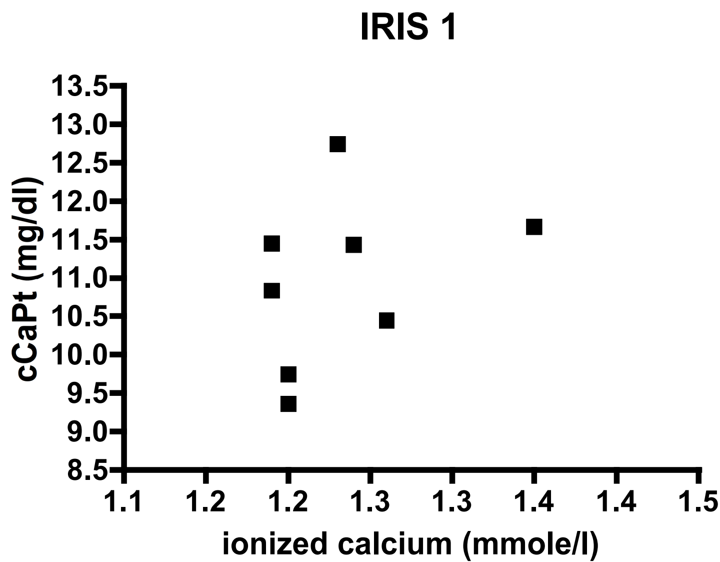

Figura 26: Pearson correlation analysis between ionized calcium (mmole/l) and cCaPt (mg/dl) in IRIS 1 dogs. No significant correlation was found. Pearson r: 0.34, P value (two-tailed): 0.18, R^2: 0.12

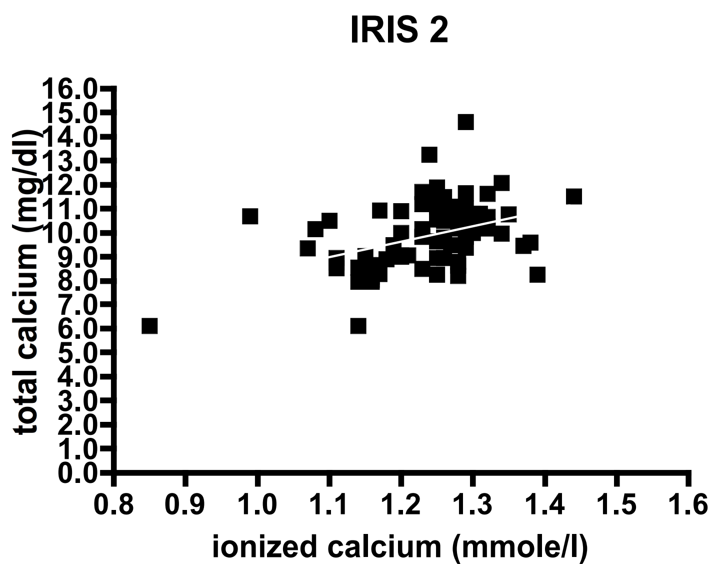

Figura 27: Pearson correlation analysis between ionized calcium (mmole/l) and total calcium (mg/dl) in IRIS 2 dogs. A significant correlation was found. Pearson r: 0.42, P value (two-tailed): 0.00, R^2: 0.18

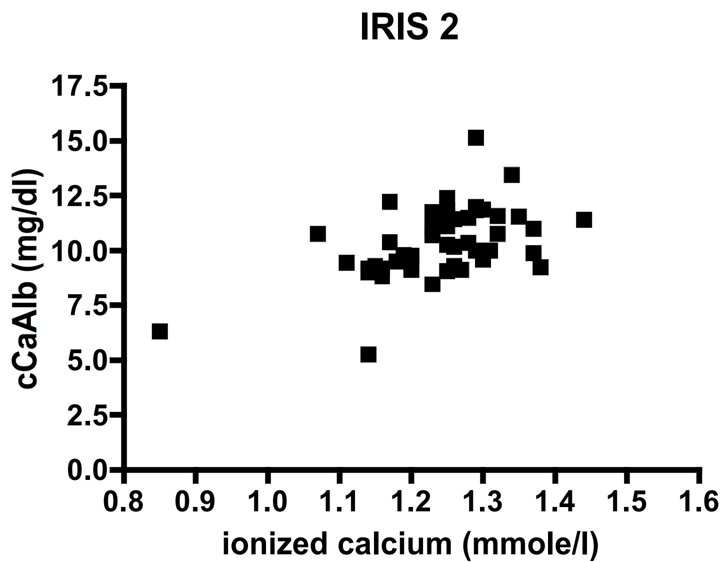

Figura 28: Pearson correlation analysis between ionized calcium (mmole/l) and tCaAlb (mg/dl) in IRIS 2 dogs. A significant correlation was found. Pearson r: 0.52, P value (two-tailed): 0.00, R^2: 0.27

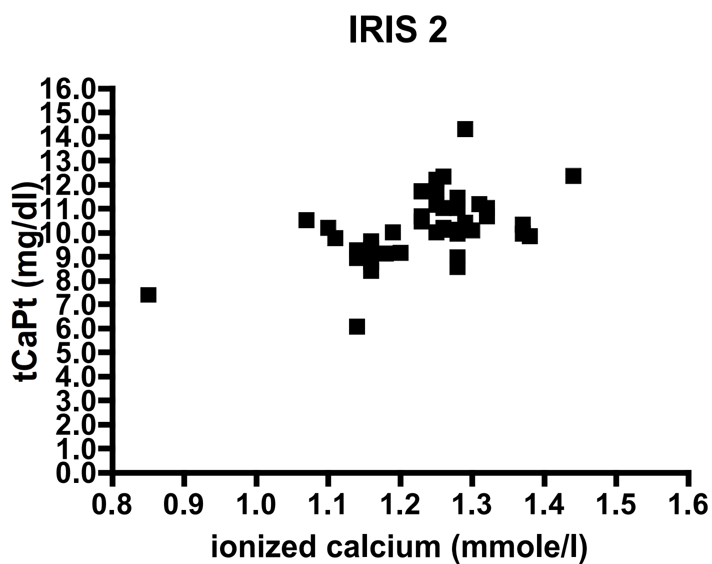

Figura 29: Pearson correlation analysis between ionized calcium (mmole/l) and tCaPt (mg/dl) in IRIS 2 dogs. A significant correlation was found. Pearson r: 0.51, P value (two-tailed): 0.00, R^2: 0.26

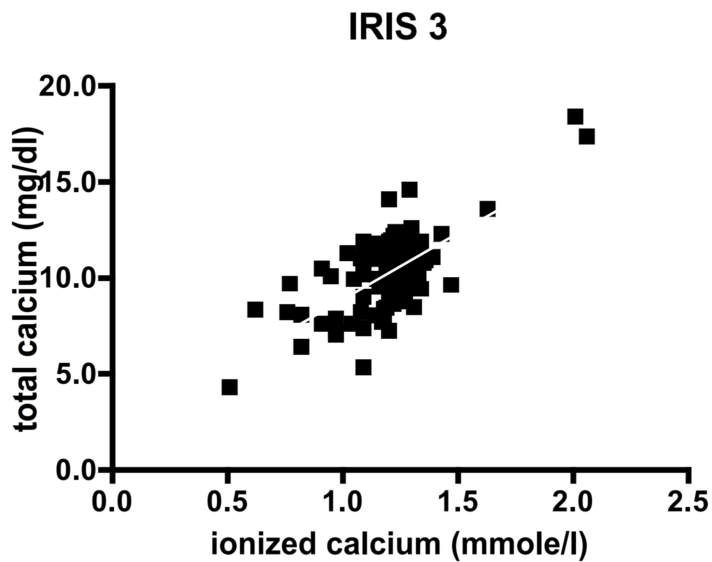

Figura 30: Pearson correlation analysis between ionized calcium (mmole/l) and total calcium (mg/dl) in IRIS 3 dogs. A significant correlation was found. Pearson r: 0.68, P value (two-tailed): 0.00, R^2: 0.47

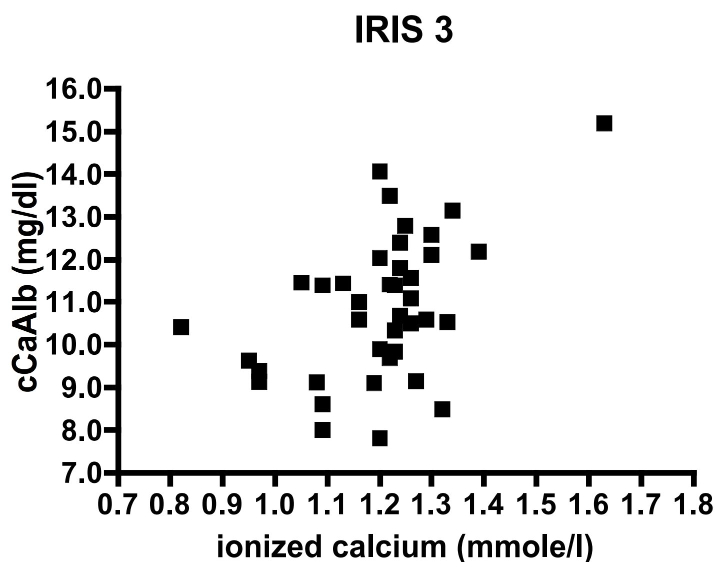

Figura 31: Pearson correlation analysis between ionized calcium (mmole/l) and cCaAlb (mg/dl) in IRIS 3 dogs. A significant correlation was found. Pearson r: 0.49, P value (two-tailed): 0.00, R^2: 0.24

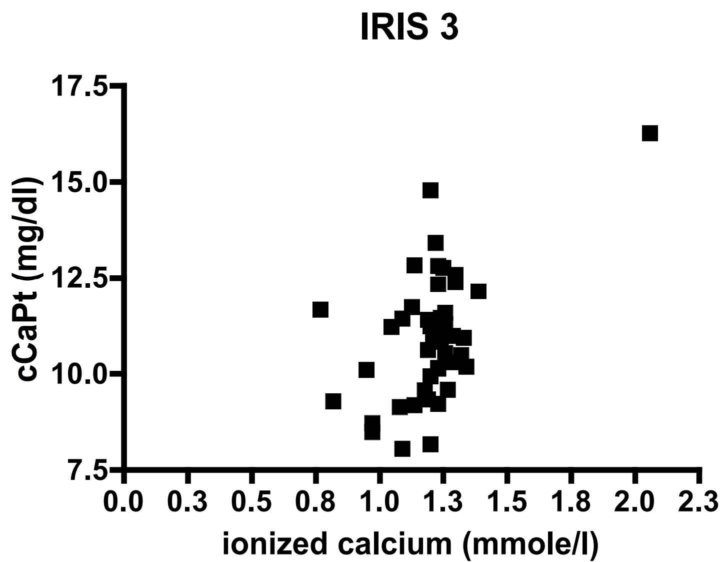

Figura 32: Pearson correlation analysis between ionized calcium (mmole/l) and cCaPt (mg/dl) in IRIS 3 dogs. A significant correlation was found. Pearson r: 0.52, P value (two-tailed): 0.00, R^2: 0.28

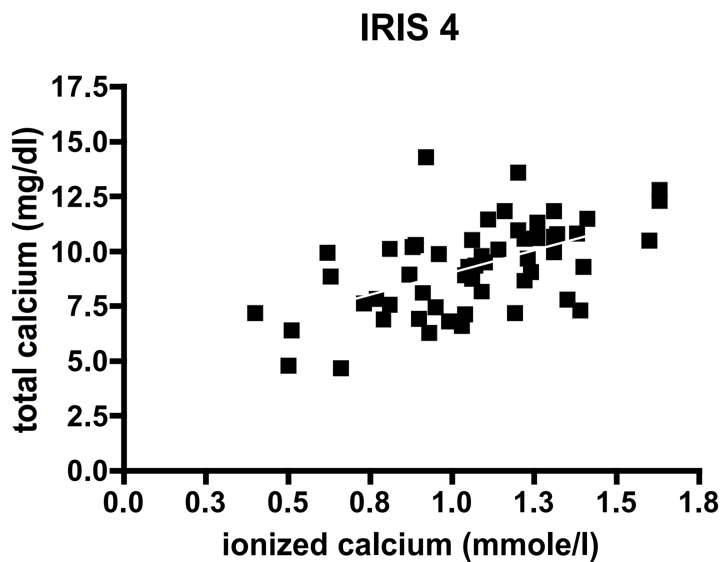

Figura 33: Pearson correlation analysis between ionized calcium (mmole/l) and total calcium (mg/dl) in IRIS 4 dogs. A significant correlation was found. Pearson r: 0.25, P value (two-tailed): 0.00, R^2: 0.06

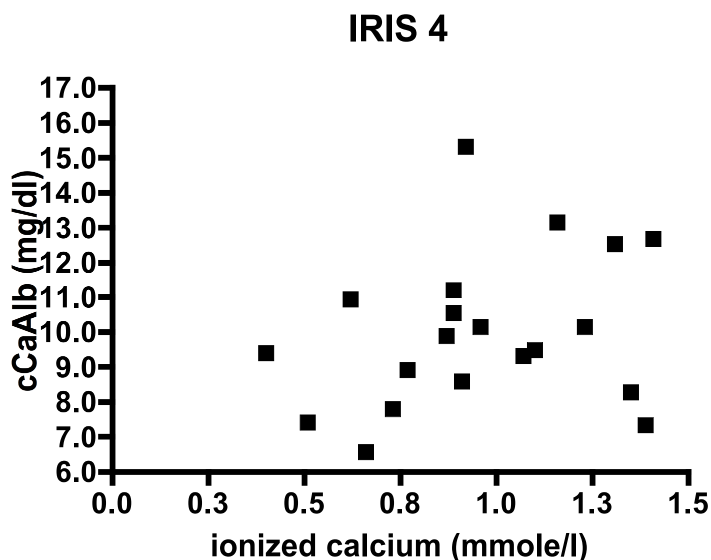

Figura 34: Pearson correlation analysis between ionized calcium (mmole/l) and cCaAlb (mg/dl) in IRIS 4 dogs. No significant correlation was found. Pearson r: 0.01, P value (two-tailed): 0.82, R^2: 0.00

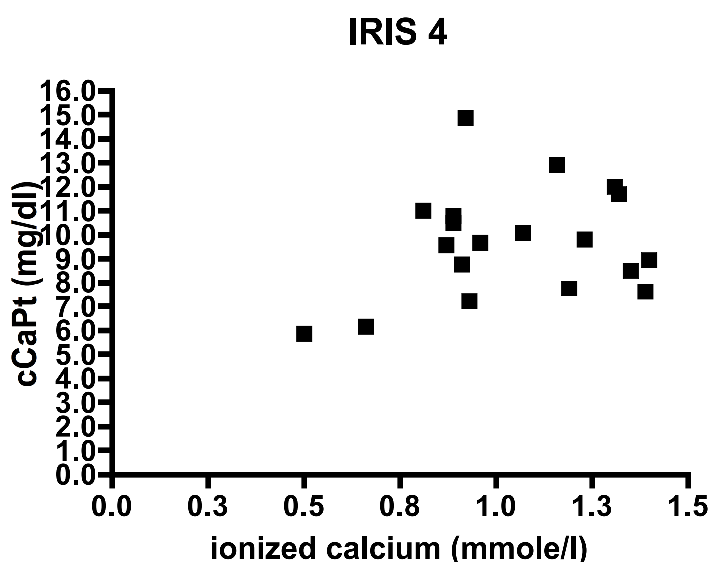

Figura 35: Pearson correlation analysis between ionized calcium (mmole/l) and cCaAlb (mg/dl) in IRIS 4 dogs. No significant correlation was found. Pearson r: 0.14, P value (two-tailed): 0.09, R^2: 0.02

In CH patients, Pearson test ($p<0.05$) between ionized calcium and total calcium showed a significant correlation ($p=0.00$), Pearson test ($p<0.05$) between ionized calcium and cCaAlb showed no significant correlation ($p=0.82$), Pearson test ($p<0.05$) between ionized calcium and cCaPt showed no significant correlation ($p=0.09$).

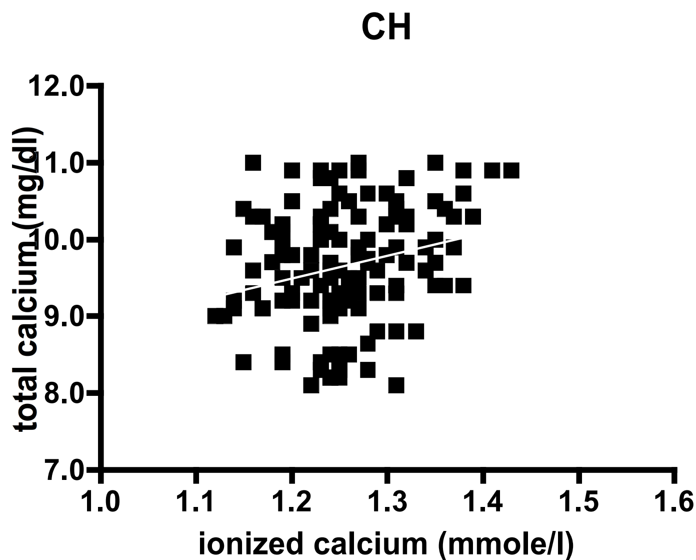

Figura 36: Pearson correlation analysis between ionized calcium (mmole/l) and total calcium (mg/dl) in CH dogs. No significant correlation was found. Pearson r: 0.25, P value (two-tailed): 0.00, R^2: 0.06

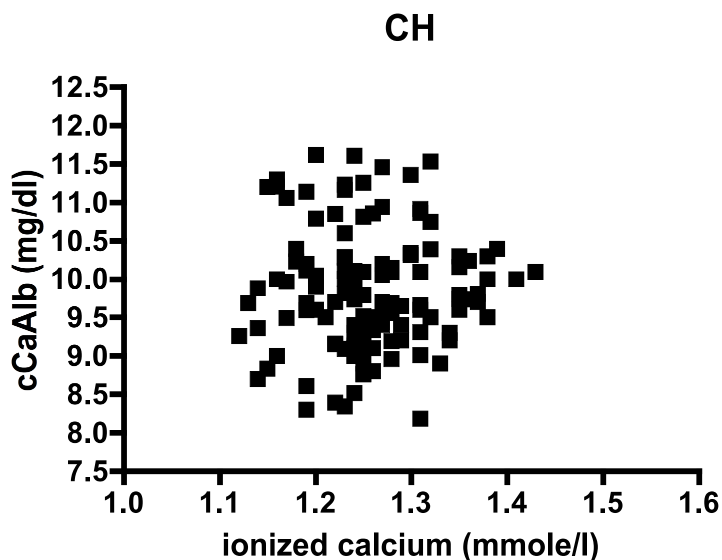

Figura 37: Pearson correlation analysis between ionized calcium (mmole/l) and cCaAlb (mg/dl) in CH dogs. No significant correlation was found. Pearson r: 0.01, P value (two-tailed): 0.82, R^2: 0.00

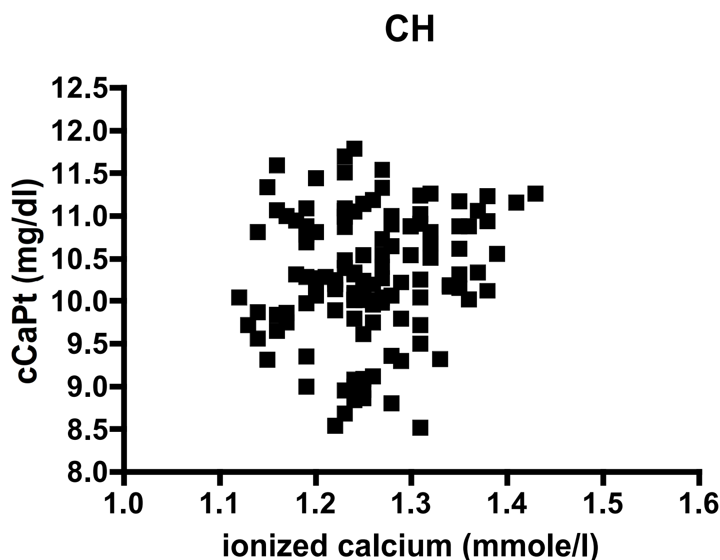

Figura 38: Pearson correlation analysis between ionized calcium (mmole/l) and cCaPt (mg/dl) in CH dogs. No significant correlation was found. Pearson r: 0.14, P value (two-tailed): 0.09, R^2: 0.02

Ca x P product (Ca x P)

Calcium x phosphate product (Ca x P) was obtained by multiplying serum total calcium (mg/dl) per phosphate (mg/dl). Ca x P was calculated in 22 IRIS 1, 83 IRIS 2, 130 IRIS 3 and 66 IRIS 4 CKD patients and in 20 CH subjects (control group). Ca x P in CH subjects ranged from a minimum of 24.00 to a maximum of 36.00, showing a mean value of 30.22 ± 0.85 SE. Ca x P in IRIS 1 subjects ranged from a minimum of 25.20 to a maximum of 70.20, showing a mean value of 39.06 ± 3.03 SE. Ca x P in IRIS 2 subjects ranged from a minimum of 17.08 to a maximum of 47.97, showing a mean value of 34.48 ± 4.73 SE. Ca x P in IRIS 3 subjects ranged from a minimum of 23.80 to a maximum of 150.50, showing a mean value of 77.92 ± 10.17 SE. Ca x P in IRIS 4 subjects ranged from a minimum of 80.64 to a maximum of 275.20, showing a mean value of 195.80 ± 126.20 SE.

One-way ANOVA ($p<0.05$) among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients showed a significant difference in the mean value of Ca x P ($p<0.0001$ and $R^2=0.75$). Tukey's post-test showed that mean Ca x P of CH subjects was significantly different ($p<0.001$) from Ca x P of IRIS 3 and IRIS 4 patients. Mean Ca x P of IRIS 1 patients was significantly different from Ca x P of IRIS 3 ($p<0.05$) and IRIS 4 ($p<0.001$) patients. Mean Ca x P of IRIS 2 patients was significantly different from Ca x P of IRIS 3 ($p<0.05$) and IRIS 4 ($p<0.001$) patients. Mean Ca x P of IRIS 3 patients was significantly different ($p<0.001$) from Ca x P of IRIS 4 patients. According to Tukey's post-test, no significant difference was found between CH and IRIS 1 subjects, CH and IRIS 2 subjects, IRIS 1 and IRIS 2 patients.

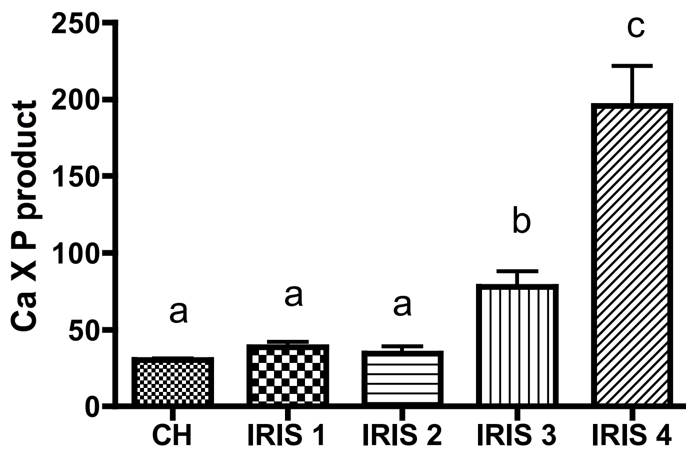

Figura 39: One-way ANOVA analysis of variance of mean Ca x P among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients. A significant difference in mean Ca x P among different groups was found ($p<0.0001$, $R^2=0.75$). Tukey's post-test showed a significant difference among CH, IRIS 1, IRIS 2 (a) and IRIS 3 (b) and IRIS 4 (c)

ANOVA test Ca x P	
One-way analysis of variance	
P value	<0.0001
P value summary	***
Are means signif. different? (p<0.05)	Yes
Number of groups	5
F	46.33
R^2	0.75

Tukey's test	Mean diff	q	P value	95% Cl of diff
CH vs IRIS 1	-8.84	1.09	>0.05	-40.97 to 23.29
CH vs IRIS 2	-4.26	0.42	>0.05	-43.87 to 35.35
CH vs IRIS 3	-47.70	5.92	<0.001	-79.83 to -15.57
CH vs IRIS 4	-165.50	17.50	<0.001	-203.3 to -127.8
IRIS 1 vs IRIS 2	4.58	0.43	>0.05	-37.70 to 46.87
IRIS 1 vs IRIS 3	-38.85	4.38	<0.05	-74.23 to -3.478
IRIS 1 vs IRIS 4	-156.70	15.42	<0.001	-197.2 to -116.2
IRIS 2 vs IRIS 3	-43.44	4.09	<0.05	-85.72 to -1.156
IRIS 2 vs IRIS 4	-161.30	13.78	<0.001	-208.0 to -114.6
IRIS 3 vs IRIS 4	-117.80	11.60	<0.001	-158.4 to -77.32

Pearson correlation analysis ($p<0.05$) between plasma creatinine and Ca x P showed no significant correlation in IRIS 1 ($p=0.928$, $R^2=0.00$), IRIS 2 ($p=0.969$, $R^2=0.00$), IRIS 3 ($p=0.541$, $R^2=0.03$) and IRIS 4 ($p=0.06$, $R^2=0.49$) patients.

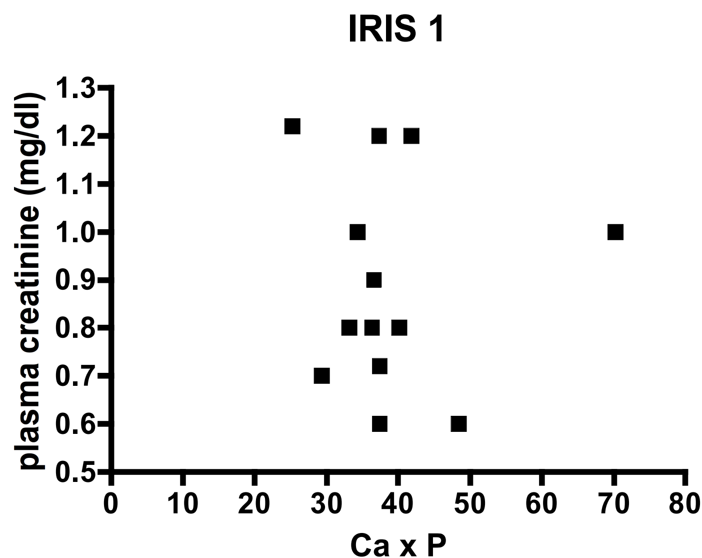

Figura 40: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and Ca x P in 13 CKD subjects belonging to IRIS 1. No significant correlation between plasma creatinine and Ca x P was found ($p=0.928$ and $R^2=0.00$)

PEARSON CORRELATION ANALYSIS	IRIS 1
Number of XY Pairs	13
Pearson r	-0,02784
95% confidence interval	-0.5702 to 0.5314
P value (two-tailed)	0,9281
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,0007752

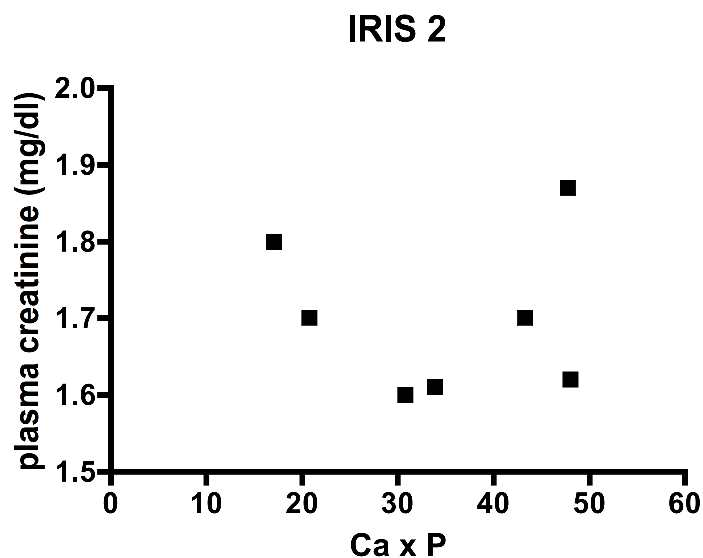

Figura 41: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and Ca x P in 7 CKD subjects belonging to IRIS 2. No significant correlation between plasma creatinine and Ca x P was found ($p=0.969$ and $R^2=0.00$)

PEARSON CORRELATION ANALYSIS	IRIS 2
Number of XY Pairs	7
Pearson r	-0,01801
95% confidence interval	-0.7609 to 0.7453
P value (two-tailed)	0,9694
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,0003242

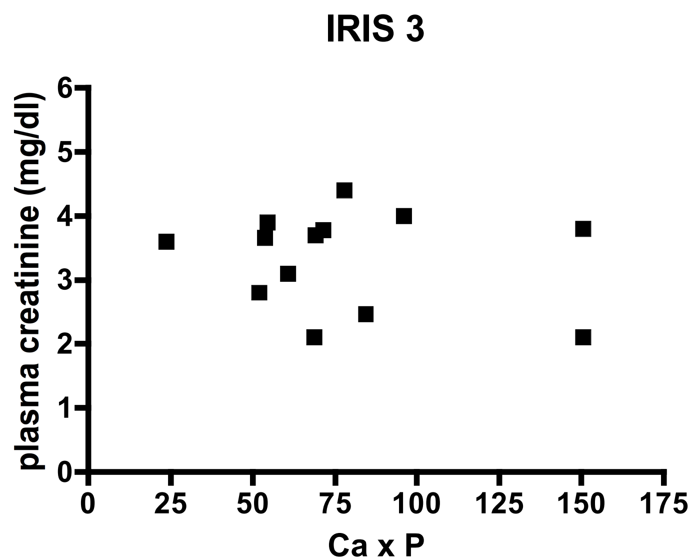

Figura 42: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and Ca x P in 13 CKD subjects belonging to IRIS 3. No significant correlation between plasma creatinine and Ca x P was found ($p=0.541$ and $R^2=0.03$)

PEARSON CORRELATION ANALYSIS	IRIS 3
Number of XY Pairs	13
Pearson r	-0,1865
95% confidence interval	-0.6689 to 0.4063
P value (two-tailed)	0,5417
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,03480

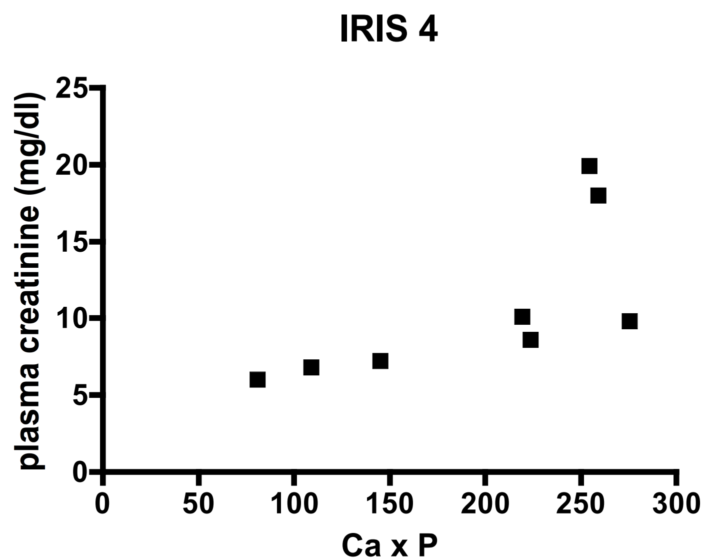

Figura 43: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and Ca x P in 11 CKD subjects belonging to IRIS 4. No significant correlation between plasma creatinine and Ca x P was found ($p=0.06$ and $R^2=0.49$)

PEARSON CORRELATION ANALYSIS	IRIS 4
Number of XY Pairs	11
Pearson r	0,7028
95% confidence interval	-0.003866 to 0.9413
P value (two-tailed)	0,0519
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,4940

Pearson correlation analysis ($p<0.05$) between plasma creatinine and Ca x P showed no significant correlation in CH ($p=0.213$, $R^2=0.08$) subjects.

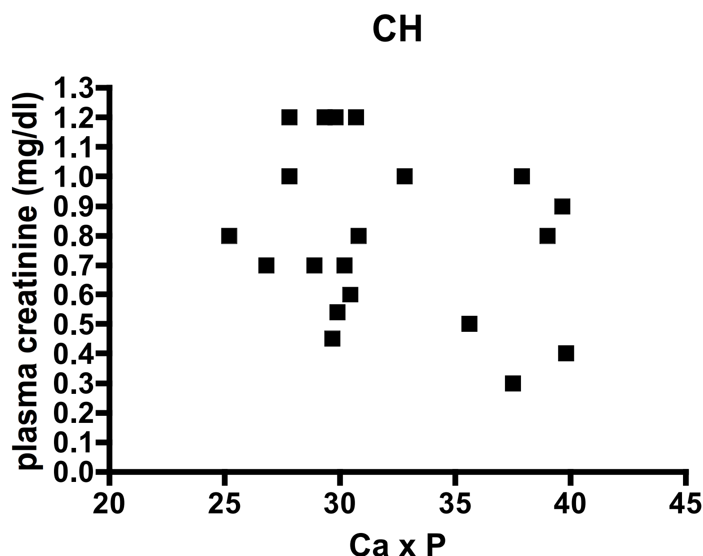

Figura 44: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and Ca x P in 20 CH subjects. No significant correlation between plasma creatinine and Ca x P was found ($p=0.213$ and $R^2=0.08$)

PEARSON CORRELATION ANALYSIS	CH
Number of XY Pairs	20
Pearson r	-0,2908
95% confidence interval	-0.6498 to 0.1743
P value (two-tailed)	0,2136
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,08455

Contingency analysis ($p<0.05$), through χ^2 test, between the number of subjects with Ca x P above and below 70 in CH subjects and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients showed a significant difference ($p<0.0001$). All 20 subjects of CH group showed Ca x P below 70. In IRIS 1 group, 12 patients showed Ca x P below 70, while 1 patient showed Ca x P above 70. In IRIS 2 group, all 7 patients showed Ca x P below 70. In IRIS 3 group, 9 patients showed Ca x P below 70, while 4 patient showed Ca x P above 70. In IRIS 4 group, all 11 patients showed Ca x P above 70.

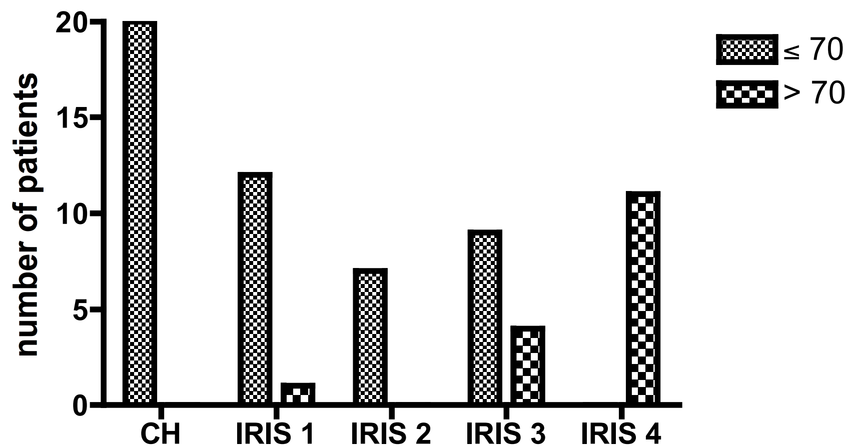

Figura 45: contingency analysis ($p<0.05$) between the number of subject with Ca x P below and above 70 in CH and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients, showed a significant difference ($p<0.001$)

χ^2 TEST	CTNI
Chi-square, df	44.31, 4
P value	P<0.0001
P value summary	***
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	Yes

Contingency analysis ($p<0.05$), through χ^2 test, between the number of survived subjects and dead subjects in the group of CKD patients with Ca x P above and below 70 showed a significant difference ($p<0.0008$). In the group of subjects with Ca x P below 70, 17 dogs survived, while 10 died after a period of time of 455 days. In the group of subjects with Ca x P above 70, only 2 dogs survived, while 15 died after a period of time of 455 days.

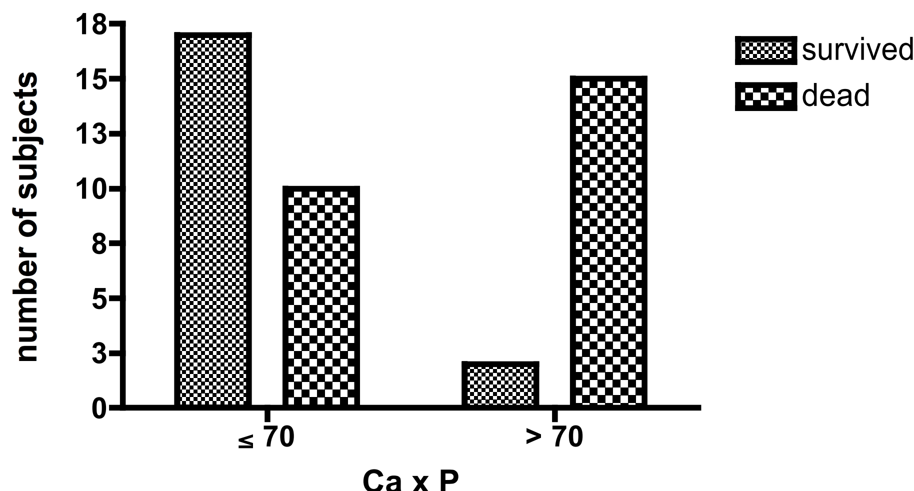

Figura 46: contingency analysis, through χ^2 test, of survived and dead CKD patients in the group of dogs with Ca x P below and above 70. A significant difference (p<0.0008) between the groups of subjects was found. In group with Ca x P below 70, 17 dogs survived while 10 died. In group with Ca x P above 70, 2 dogs survived while 15 died.

χ^2 TEST	CA X P
Chi-square, df	11.15, 1
P value	0,0008
P value summary	***
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	Yes

Log-rank test (p<0.05) between the group of subjects with Ca x P below 70 and the group of subjects with Ca x P above 70 showed a significant difference (p<0.0002). In the group of patients with Ca x P below 70, 18 dogs survived and 9 died in a 455 day period, while in the group of patients with Ca x P above 70, only 2 dogs survived while 15 died. Kaplan-Meier survival curve showed a significantly higher percentage of survival (p<0.0002) of CKD patients with Ca x P below 70, compared to patients with Ca x P above 70.

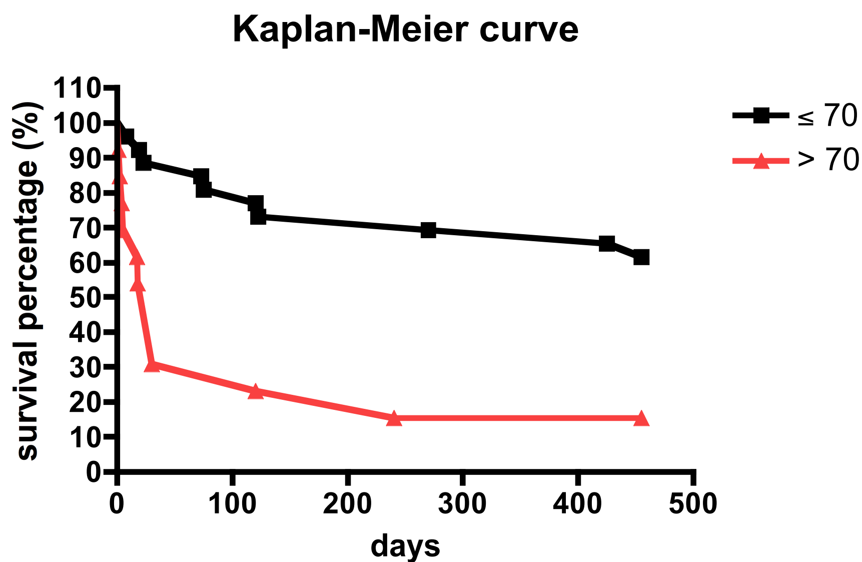

Figura 47: Kaplan-Meier survival curve between the group of CKD patients with Ca x P below 70 and the group of patients with Ca x P above 70. Patients with Ca x P below 70 showed a significantly higher percentage of survival ($p<0.0002$) compared to the other group

Cardiac troponin I (cTnI)

Cardiac troponin I (cTnI) was determined in 13 IRIS 1, 7 IRIS 2, 13 IRIS 3 and 11 IRIS 4 CKD patients and in 20 CH subjects (control group). All CH subjects showed a serum cTnI below 0.20 ng/ml. In IRIS 1 patients, 4/13 subjects presented cTnI above the reference range, with a mean value of 0.98 ± 0.28 ng/ml, ranging from a minimum of 0.43 ng/ml and a maximum of 1.66 ng/ml. In IRIS 2 patients, 4/7 subjects presented cTnI above the reference range, with a mean value of 6.90 ± 6.36 ng/ml, ranging from a minimum of 0.24 ng/ml and a maximum of 26.00 ng/ml. In IRIS 3 patients, 11/13 subjects presented cTnI above the reference range, with a mean value of 6.19 ± 3.99 ng/ml, ranging from a minimum of 0.22 ng/ml and a maximum of 41.50 ng/ml. In IRIS 4 patients, 10/11 subjects presented cTnI above the reference range, with a mean value of 11.80 ± 5.15 ng/ml, ranging from a minimum of 0.43 ng/ml and a maximum of 51.10 ng/ml.

One-way ANOVA ($p<0.05$) among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients showed a significant difference in the mean value of cTnI ($p<0.02$ and $R^2=0.16$). Tukey’s post-test showed that mean cTnI of CH subjects was significantly different ($p<0.05$) from cTnI of IRIS 4. According to Tukey’s post-test, no significant difference was found among CH and IRIS 1, IRIS 2 and IRIS 3 subjects, among IRIS 1 and IRIS 2, IRIS 3 and IRIS 4 subjects, among IRIS 2, IRIS 3 and IRIS 4, among IRIS 3 and IRIS 4 subjects.

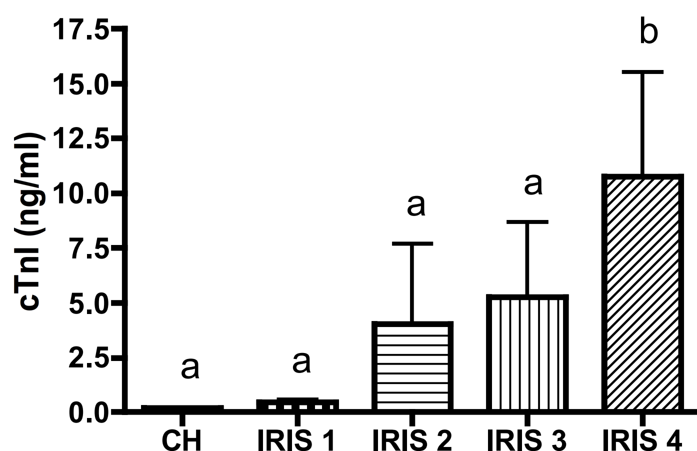

Figura 48: One-way ANOVA analysis of variance of mean cTnI among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients. A significant difference in mean cTnI among different groups was found ($p<0.02$), $R^2=0.16$). Tukey’s post-test showed a significant difference among CH (a) and IRIS 4 (b) patients

ANOVA test	cTnI
One-way analysis of variance	
P value	0,0297
P value summary	*
Are means signif. different? ($p<0.05$)	Yes
Number of groups	5
F	2,892
R^2	0,1639

Tukey's test	Mean diff	q	P value	95% Cl of diff
CH vs IRIS 1	-0,2408	0,1049	P > 0.05	-9.373 to 8.892
CH vs IRIS 2	-3,830	1,354	P > 0.05	-15.09 to 7.427
CH vs IRIS 3	-5,069	2,209	P > 0.05	-14.20 to 4.063
CH vs IRIS 4	-10,55	4,362	P < 0.05	-20.17 to -0.923
IRIS 1 vs IRIS 2	-3,589	1,189	P > 0.05	-15.61 to 8.428
IRIS 1 vs IRIS 3	-4,828	1,911	P > 0.05	-14.88 to 5.226
IRIS 1 vs IRIS 4	-10,31	3,906	P > 0.05	-20.81 to 0.1961
IRIS 2 vs IRIS 3	-1,239	0,4104	P > 0.05	-13.26 to 10.78
IRIS 2 vs IRIS 4	-6,716	2,157	P > 0.05	-19.11 to 5.678
IRIS 3 vs IRIS 4	-5,477	2,076	P > 0.05	-15.98 to 5.025

Pearson correlation analysis ($p<0.05$) between plasma creatinine and cTnI showed no significant correlation in CH subjects ($p=0.99$, $R^2=0.00$) and in IRIS 1 ($p=0.06$, $R^2=0.23$), IRIS 2 ($p=0.54$, $R^2=0.07$), IRIS 3 ($p=0.07$, $R^2=0.26$) and IRIS 4 ($p=0.57$, $R^2=0.03$) patients.

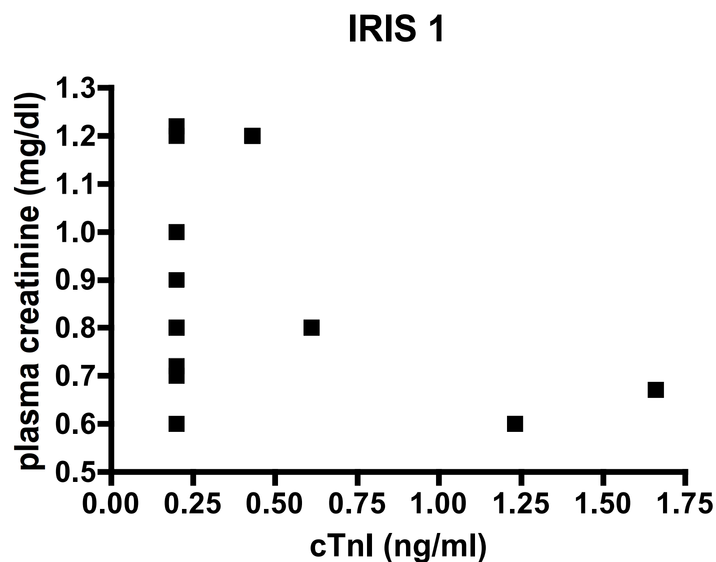

Figura 49: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and cTnI (ng(ml) in 13 IRIS 1 patients. No significant correlation between plasma creatinine and cTnI was found ($p=0.06$ and $R^2=0.23$)

PEARSON CORRELATION ANALYSIS	IRIS 1
Number of XY Pairs	13
Pearson r	-0,4804
95% confidence interval	-0.7808 to 0.0004068
P value (two-tailed)	0,0509
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,2308

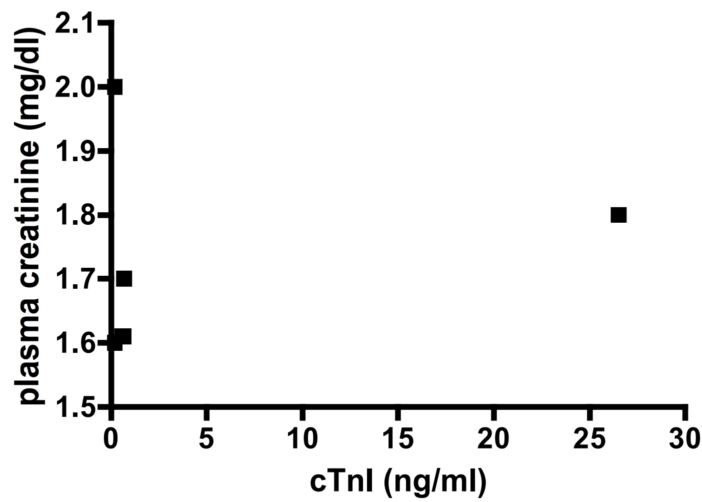

Figura 50: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and cTnI (ng/ml) in 7 IRIS 2 patients. No significant correlation between plasma creatinine and cTnI was found ($p=0.54$ and $R^2=0.07$)

PEARSON CORRELATION ANALYSIS	IRIS 2
Number of XY Pairs	7
Pearson r	0,2774
95% confidence interval	-0.6014 to 0.8525
P value (two-tailed)	0,5469
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,07697

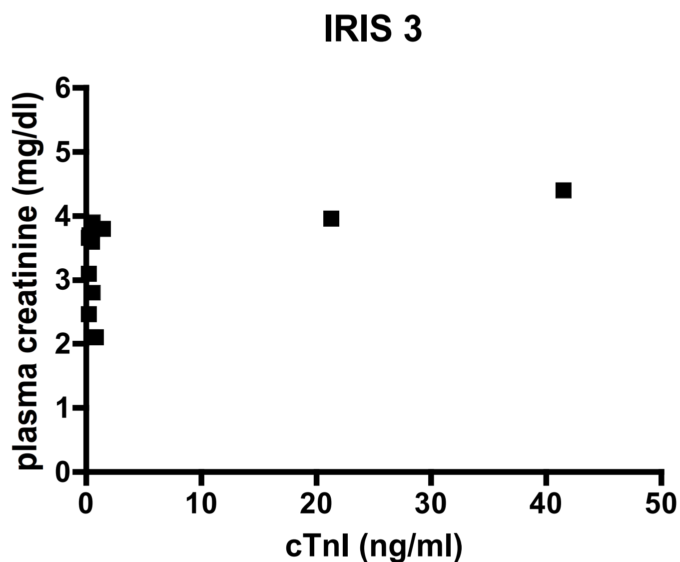

Figura 51: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and cTnI (ng/ml) in 13 IRIS 3 patients. No significant correlation between plasma creatinine and cTnI was found ($p=0.07$ and $R^2=0.26$)

PEARSON CORRELATION ANALYSIS	IRIS 3
Number of XY Pairs	13
Pearson r	0,5138
95% confidence interval	-0.05199 to 0.8299
P value (two-tailed)	0,0725
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,2640

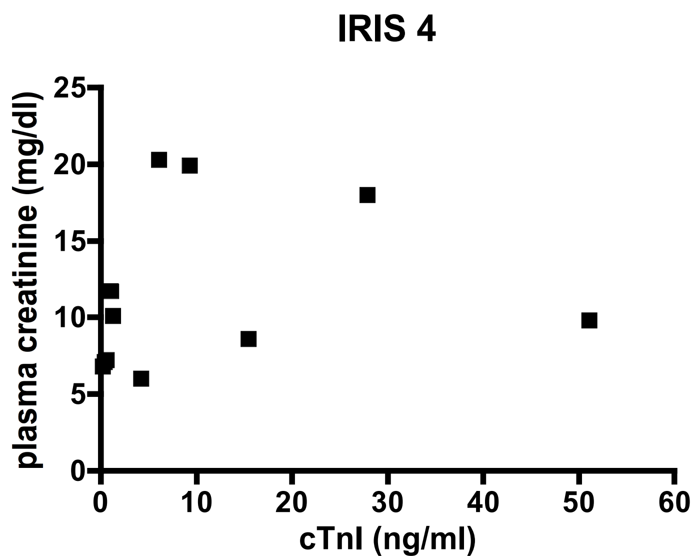

Figura 52: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and cTnI (ng/ml) in 11 IRIS 4 patients. No significant correlation between plasma creatinine and cTnI was found ($p=0.57$ and $R^2=0.03$)

PEARSON CORRELATION ANALYSIS	IRIS 4
Number of XY Pairs	11
Pearson r	0,1882
95% confidence interval	-0.4642 to 0.7082
P value (two-tailed)	0,5794
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,03542

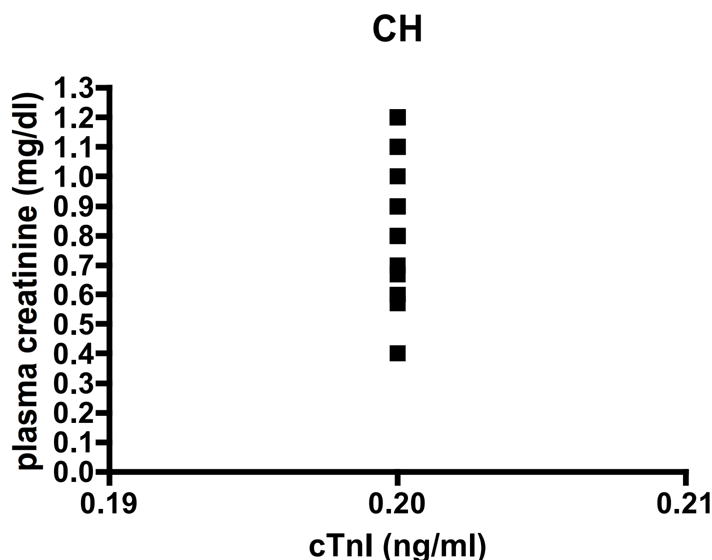

Figura 53: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and cTnI (ng/ml) in 20 CH subjects. No significant correlation between plasma creatinine and cTnI was found ($p=0.99$ and $R^2=0.00$)

PEARSON CORRELATION ANALYSIS	CH
Number of XY Pairs	20
Pearson r	0.00001935
95% confidence interval	-0.4426 to 0.4426
P value (two-tailed)	0.9999
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0.000000000375

Contingency analysis ($p<0.05$), through χ^2 test, between the number of subjects with cTnI above and below 0.20 (ng/ml) in CH subjects and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients showed a significant difference ($p=0.0001$). All 20 subjects of CH group showed cTnI below 0.20 (ng/ml). In IRIS 1 group, 9 patients showed cTnI below 0.20 (ng/ml), while 4 patients showed cTnI above 0.20 (ng/ml). In IRIS 2 group, 4 patients showed cTnI below 0.20 (ng/ml), while 3 patients showed cTnI above 0.20 (ng/ml). In IRIS 3 group, 2 patients showed cTnI below 0.20 (ng/ml), while 10 patients showed cTnI above 0.20 (ng/ml). In IRIS 4 group, only 1 patient showed cTnI below 0.20, while 10 patients showed cTnI above 0.20 (ng/ml).

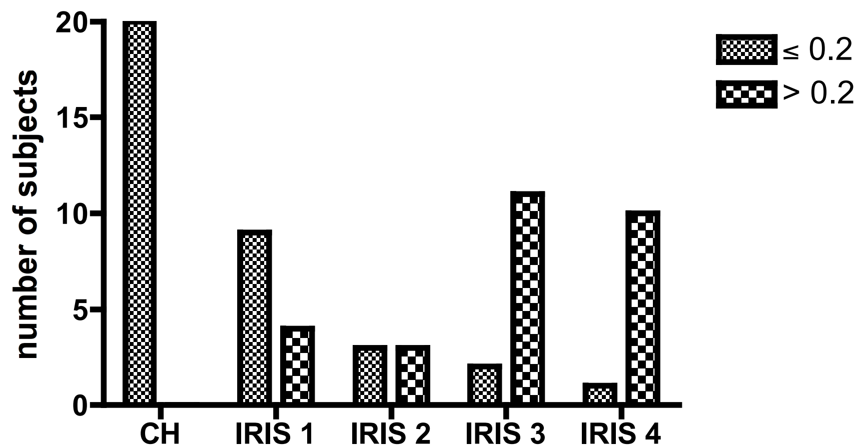

Figura 54: contingency analysis ($p<0.05$) between the number of subject with cTnI below and above 0.20 ng/ml in CH and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients, showed a significant difference ($p=0.0001$)

χ^2 TEST	cTnI
Chi-square, df	35.17, 4
P value	P<0.0001
P value summary	***
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	Yes

Contingency analysis ($p<0.05$), through χ^2 test, between the number of survived subjects and dead subjects in the group of CKD patients with cTnI above and below 0.20 ng/ml showed a significant difference ($p=0.02$). In the group of subjects with cTnI below 0.20 ng/ml, 10 dogs survived, while 5 died after a period of time of 455 days. In the group of subjects with cTnI above 0.20 ng/ml, only 7 dogs survived, while 21 died after a period of time of 455 days.

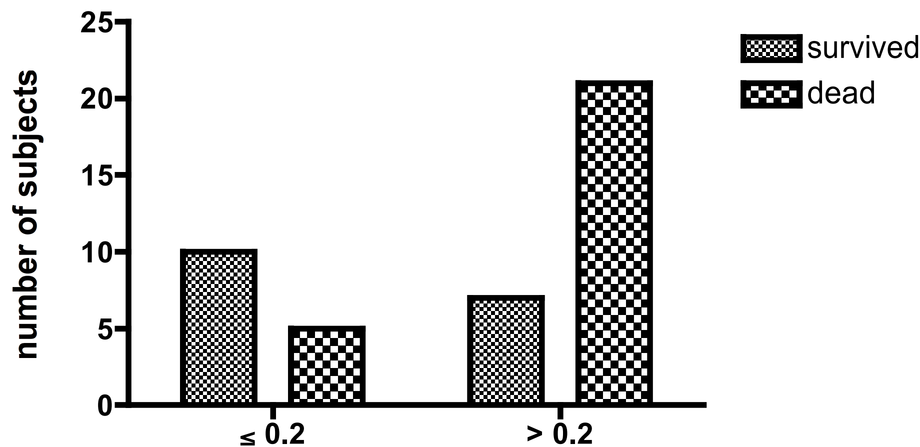

Figura 55: contingency analysis, through χ^2 test, of survived and dead CKD patients in the group of dogs with cTnI below and above 0.20 ng/ml. A significant difference (p=0.007) between the groups of subjects was found. In group with cTnI below 0.20 ng/ml, 10 dogs survived while 5 died. In group with cTnI above 0.20 ng/ml, 7 dogs survived while 21 died.

χ^2 TEST	CTNI
Chi-square, df	7.094, 1
P value	0,0077
P value summary	**
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	Yes

Log-rank test (p<0.05) between the group of subjects with cTnI below 0.20 ng/ml and the group of subjects with cTnI above 0.20 ng/ml showed a significant difference (p<0.0002). In the group of patients with cTnI below 0.20 ng/ml, 12 dogs survived and 5 died in a 455 day period, while in the group of patients with cTnI above 0.20 ng/ml, 6 dogs survived while 21 died. Kaplan-Meier survival curve showed a significantly higher percentage of survival (p<0.0007) of CKD patients with cTnI below 0.20 ng/ml compared to patients with cTnI above 0.20 ng/ml.

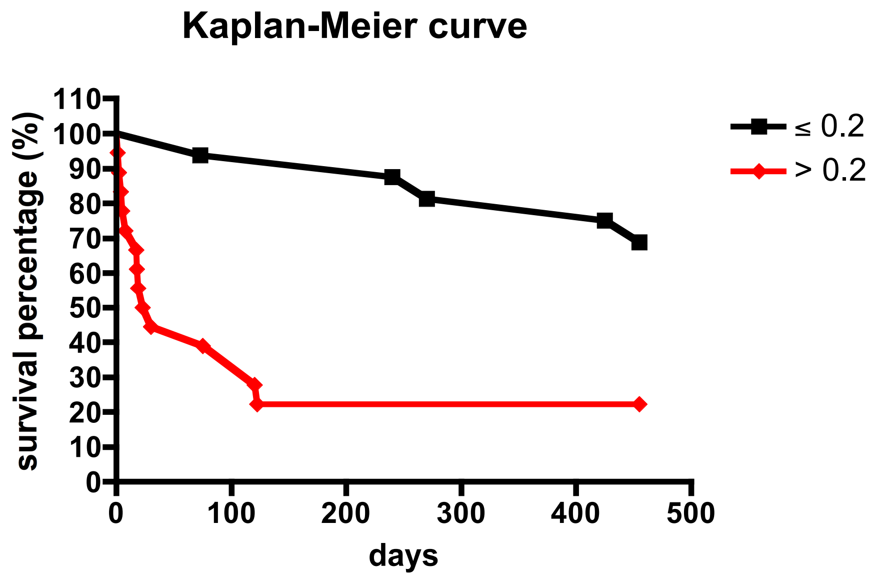

Figura 56: Kaplan-Meier survival curve between the group of CKD patients with cTnI below 0.20 ng/ml and the group of patients with cTnI above 0.20 ng/ml. Patients with cTnI below 0.20 ng/ml showed a significantly higher percentage of survival ($p<0.0007$) compared to the other group

C-reactive protein (CRP)

C-reactive protein (CRP) was determined in 13 IRIS 1, 7 IRIS 2, 13 IRIS 3 and 11 IRIS 4 CKD patients and in 20 CH subjects (control group). All CH subjects showed a serum CRP below 9.7 mg/l, with a mean value of 1.81 ± 0.36 mg/l, within a minimum of 0.00 mg/l and a maximum of 5.60 mg/l. In IRIS 1 patients, 4/13 subjects presented CRP above the reference range, while 9/13 subjects CRP below the reference range, with a mean value of 7.48 ± 2.77 mg/l, ranging from a minimum of 0.00 mg/l and a maximum of 22.40 mg/l. In IRIS 2 patients, 4/7 subjects presented CRP above the reference range, while 3/7 subjects CRP below the reference range, with a mean value of 4.28 ± 1.27 mg/l, ranging from a minimum of 0.00 mg/l and a maximum of 14.7 mg/l. In IRIS 3 patients, 6/13 subjects presented CRP above the reference range, while 7/13 subjects CRP below the reference range, with a mean value of 17.92 ± 5.16 mg/l, ranging from a minimum of 0.80 mg/l and a maximum of 54.50 mg/l. In IRIS 4 patients, 10/11 subjects presented CRP above the reference range, while only 1 patient CRP below the reference range, with a mean value of 20.15 ± 3.32 mg/l, ranging from a minimum of 1.90 mg/l and a maximum of 35 mg/l.

One-way ANOVA ($p<0.05$) among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients showed a significant difference in the mean value of CRP ($p<0.0001$ and $R^2=0.37$). Tukey's post-test showed that mean CRP of CH subjects was significantly different ($p<0.001$) from CRP of IRIS 3 and IRIS 4. Tukey's post-test showed that mean CRP of IRIS 1 subjects was significantly different ($p<0.05$) from CRP of IRIS 4 and that mean CRP of IRIS 2 was significantly different from IRIS 3 ($p<0.05$) and IRIS 4 ($p<0.01$). According to Tukey's post-test, no significant difference was found among CH, IRIS 1 and IRIS 2, among IRIS 1, IRIS 2 and IRIS 3, and between IRIS 3 and IRIS 4 subjects.

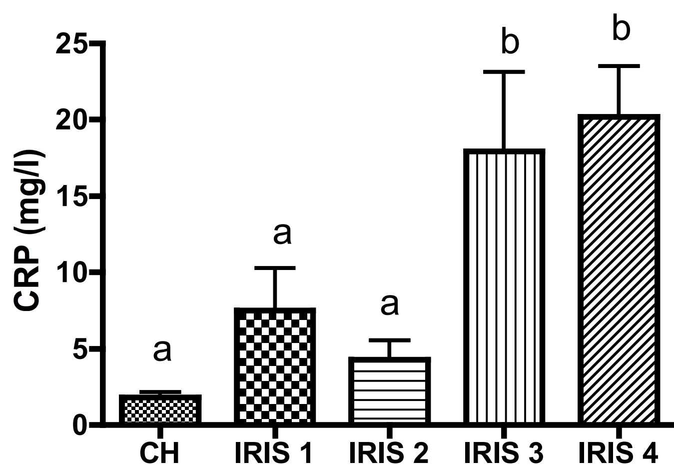

Figura 57: One-way ANOVA analysis of variance of mean CRP among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients. A significant difference in mean CRP among different groups was found ($p<0.0001$, $R^2=0.37$). Tukey's post-test showed a significant difference among CH (a), IRIS 3 and IRIS 4 (b), between IRIS 1 (a) and IRIS 4 (b) and among IRIS 2 (a), IRIS 3 (b) and IRIS 4 (b) patients

ANOVA test	**CRP**
One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (p<0.05)	Yes
Number of groups	5
F	9,423
R^2	0,3707

Tukey's test	**Mean diff**	**q**	**P value**	**95% Cl of diff**
CH vs IRIS 1	-5,668	2,153	P > 0.05	-16.13 to 4.791
CH vs IRIS 2	-2,470	0,9653	P > 0.05	-12.63 to 7.695
CH vs IRIS 3	-16,11	6,459	P < 0.001	-26.02 to -6.201
CH vs IRIS 4	-18,34	6,967	P < 0.001	-28.80 to -7.882
IRIS 1 vs IRIS 2	3,198	1,075	P > 0.05	-8.625 to 15.02
IRIS 1 vs IRIS 3	-10,44	3,575	P > 0.05	-22.04 to 1.162
IRIS 1 vs IRIS 4	-12,67	4,169	P < 0.05	-24.75 to -0.595

IRIS 2 vs IRIS 3	-13,64	4,779	P < 0.05	-24.98 to -2.301
IRIS 2 vs IRIS 4	-15,87	5,333	P < 0.01	-27.69 to -4.048
IRIS 3 vs IRIS 4	-2,231	0,7640	P > 0.05	-13.84 to 9.372

Pearson correlation analysis ($p<0.05$) between plasma creatinine and CRP showed no significant correlation in CH subjects ($p=0.34$, $R^2=0.04$) and in IRIS 1 ($p=0.08$, $R^2=0.28$), IRIS 2 ($p=0.67$, $R^2=0.01$), IRIS 3 ($p=0.22$, $R^2=0.13$) and IRIS 4 ($p=0.09$, $R^2=0.28$) patients.

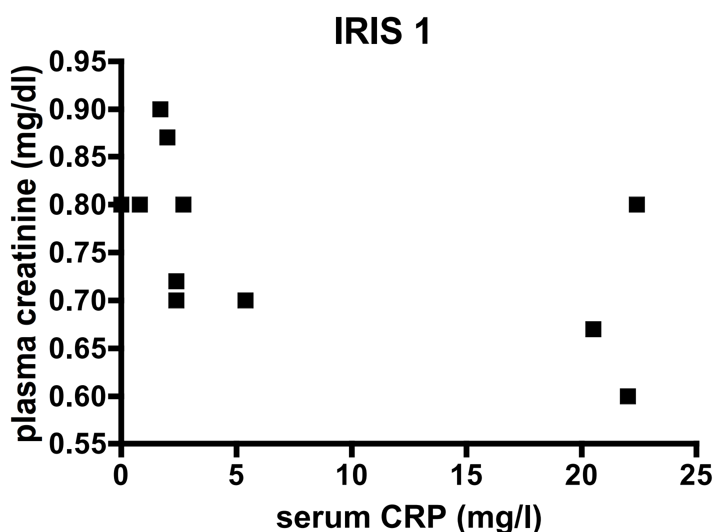

Figura 58: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and CRP (mg/l) in 13 IRIS 1 patients. No significant correlation between plasma creatinine and CRP was found ($p=0.34$ and $R^2=0.04$)

PEARSON CORRELATION ANALYSIS	IRIS 1
Number of XY Pairs	13
Pearson r	-0,5372
95% confidence interval	-0.8600 to 0.09270
P value (two-tailed)	0,0884
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,2885

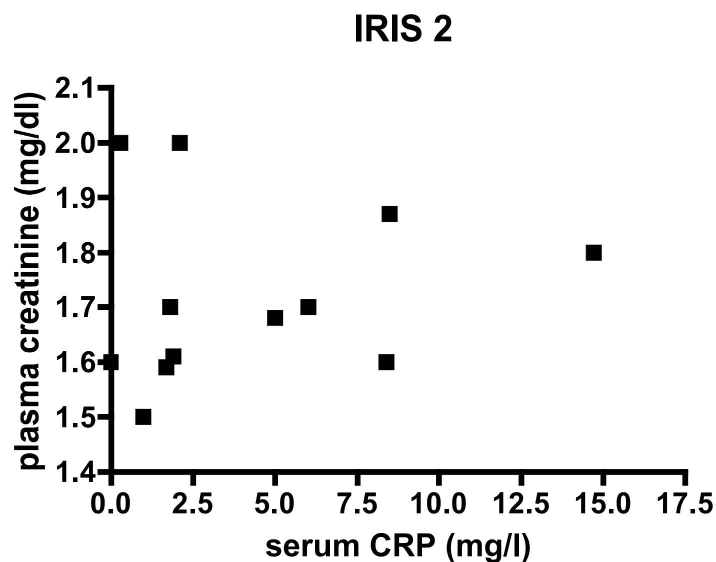

Figura 59: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and CRP (mg/l) in 7 IRIS 2 patients. No significant correlation between plasma creatinine and CRP was found ($p=0.67$ and $R^2=0.01$)

PEARSON CORRELATION ANALYSIS	IRIS 2
Number of XY Pairs	7
Pearson r	0,1340
95% confidence interval	-0.4766 to 0.6575
P value (two-tailed)	0,6779
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,01797

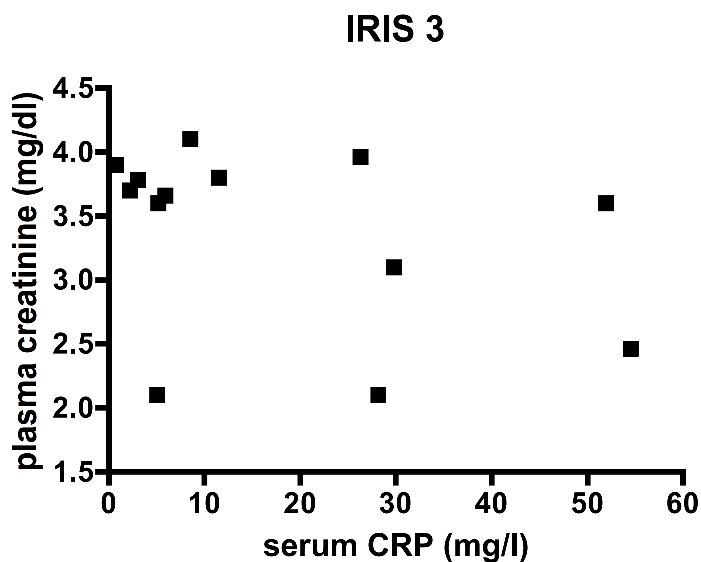

Figura 60: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and CRP (mg/l) in 13 IRIS 2 patients. No significant correlation between plasma creatinine and CRP was found ($p=0.22$ and $R^2=0.13$)

PEARSON CORRELATION ANALYSIS	IRIS 3
Number of XY Pairs	13
Pearson r	-0,3607
95% confidence interval	-0.7606 to 0.2376
P value (two-tailed)	0,2260
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,1301

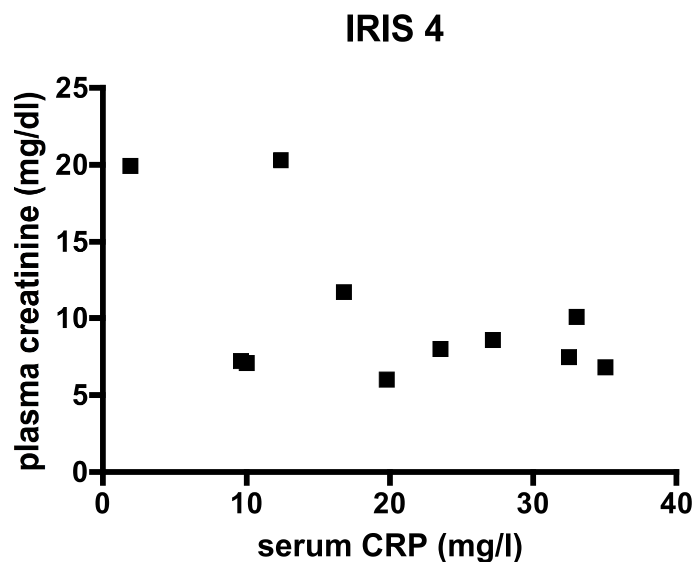

Figura 61: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and CRP (mg/l) in 11 IRIS 4 patients. No significant correlation between plasma creatinine and CRP was found ($p=0.09$ and $R^2=0.28$)

PEARSON CORRELATION ANALYSIS	IRIS 4
Number of XY Pairs	11
Pearson r	-0,5321
95% confidence interval	-0.8581 to 0.09976
P value (two-tailed)	0,0920
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,2831

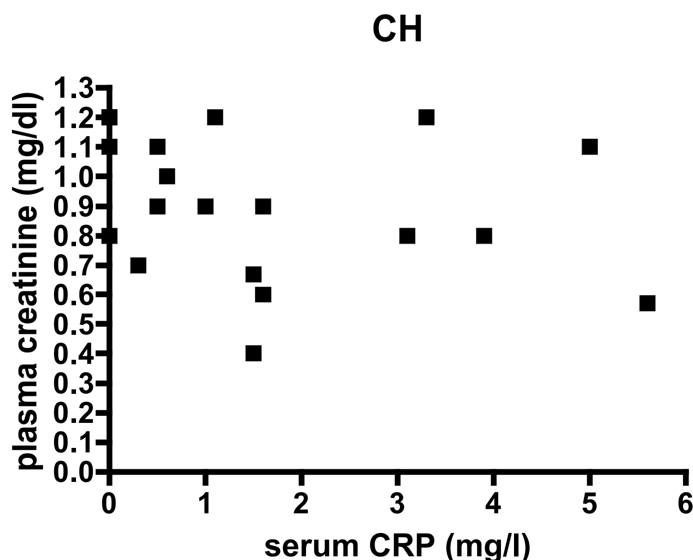

Figura 62: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and CRP (mg/l) in 7 CH subjects. No significant correlation between plasma creatinine and CRP was found ($p=0.34$ and $R^2=0.04$)

PEARSON CORRELATION ANALYSIS	CH
Number of XY Pairs	20
Pearson r	-0,2233
95% confidence interval	-0.6060 to 0.2433
P value (two-tailed)	0,3439
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,04988

Contingency analysis ($p<0.05$), through χ^2 test, between the number of subjects with CRP above and below 9.7 (mg/l) in CH subjects and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients showed a significant difference ($p=0.0001$). All 20 subjects of CH group showed CRP below 9.7 (mg/l). In IRIS 1 group, 9 patients showed CRP below 9.7 (mg/l), while 4 patients showed CRP above 9.7 (mg/l). In IRIS 2 group, 6 patients showed CRP below 9.7 (mg/l), while 1 patient showed CRP above 9.7 (mg/l). In IRIS 3 group, 7 patients showed CRP below 9.7 (mg/l), while 6 patients showed CRP above 9.7 (mg/l). In IRIS 4 group, 2 patients showed CRP below 9.7 mg/l, while 10 patients showed CRP above 9.7 (ng/ml).

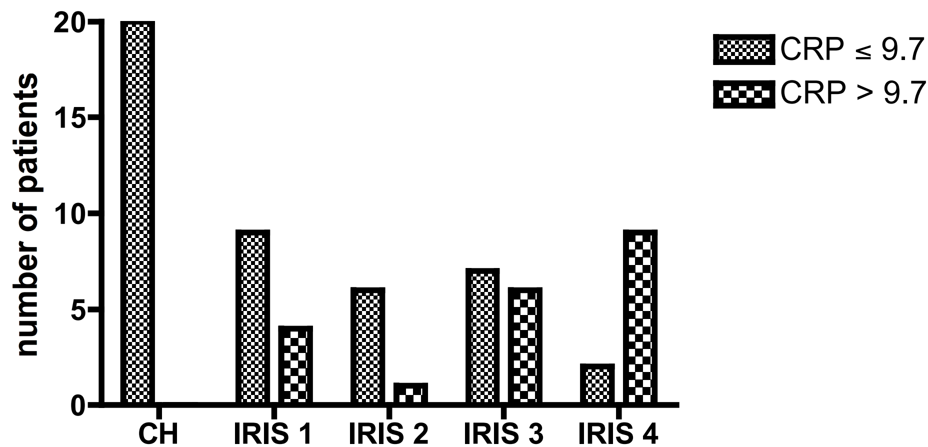

Figura 63: contingency analysis ($p<0.05$) between the number of subject with CRP below and above 9.7 mg/l in CH and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients, showed a significant difference ($p=0.02$)

χ^2 TEST	CRP
Chi-square, df	10.75, 4
P value	0,0295
P value summary	*
One- or two-sided	NA
Statistically significant? (alpha<0.05)	Yes

Contingency analysis ($p<0.05$), through χ^2 test, between the number of survived subjects and dead subjects in the group of CKD patients with CRP above and below 9.7 mg/l showed a significant difference ($p=0.0009$). In the group of subjects with CRP below 9.7 mg/l, 18 dogs survived, while 6 died after a period of time of 455 days. In the group of subjects with CRP above 9.7 mg/l, only 5 dogs survived, while 15 died after a period of time of 455 days.

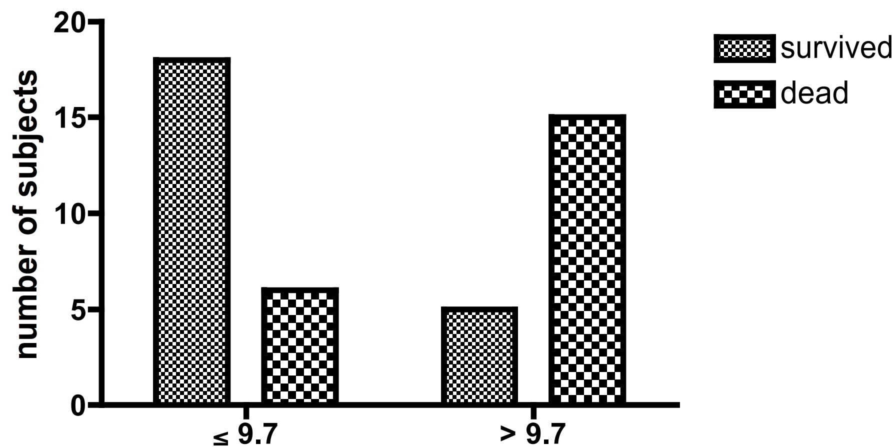

Figura 64: contingency analysis, through χ^2 test, of survived and dead CKD patients in the group of dogs with CRP below and above 9.7 mg/l. A significant difference (p=0.0009) between the groups of subjects was found. In group with CRP below 9.7, 18 dogs survived while 6 died. In group with CRP above 9.7 mg/l, 5 dogs survived while 15 died.

χ^2 TEST	CRP
Chi-square, df	10.93, 1
P value	0,0009
P value summary	***
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	Yes

Log-rank test ($p<0.05$) between the group of subjects with CRP below 9.7 mg/l and the group of subjects with CRP above 9.7 mg/l showed a significant difference ($p<0.0009$). In the group of patients with CRP below 9.7 mg/l, 18 dogs survived and 6 died in a 455 day period, while in the group of patients with CRP above 9.7 mg/l, 5 dogs survived while 15 died. Kaplan-Meier survival curve showed a significantly higher percentage of survival ($p<0.001$) of CKD patients with CRP below 9.7 mg/l compared to patients with CRP above 9.7 mg/l.

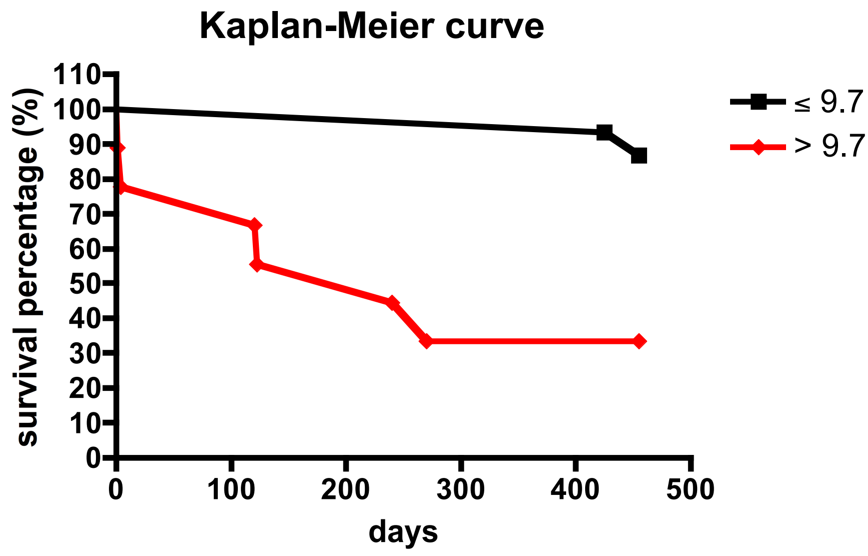

Figura 65: Kaplan-Meier survival curve between the group of CKD patients with CRP below 9.7 mg/l and the group of patients with CRP above 9.7 mg/dl. Patients with CRP below 9.7 mg/l showed a significantly higher percentage of survival (p=0.001) compared to the other group

α-tochopherol

α-tochopherol was determined in 13 IRIS 1, 7 IRIS 2, 13 IRIS 3 and 11 IRIS 4 CKD patients and in 20 CH subjects (control group). In CH group, 3/20 subjects showed serum α-tochopherol below 21.6 ppm, while 17/20 subjects showed α-tochopherol above the reference range, with a mean value of 26.56 ± 1.11 ppm, within a minimum of 17.96 ppm and a maximum of 35.88 ppm. In IRIS 1 group, 1/13 subject presented α-tochopherol above the reference range, while 12/13 subjects α-tochopherol below the reference range, with a mean value of 10.71 ± 3.93 ppm, ranging from a minimum of 0.42 ppm and a maximum of 49.84 ppm. In IRIS 2 group, 2/7 subjects presented α-tochopherol above the reference range, while 5/7 subjects α-tochopherol below the reference range, with a mean value of 17.48 ± 7.07 ppm, ranging from a minimum of 0.84 ppm and a maximum of 60.18 ppm. In IRIS 3 group, 6/13 subjects presented α-tochopherol above the reference range, while 7/13 subjects α-tochopherol below the reference range, with a mean value of 22.42 ± 3.88 ppm, ranging from a minimum of 2.49 ppm and a maximum of 48.49 ppm. In IRIS 4 group, all subjects presented α-tochopherol below the reference range, with a mean value of 6.84 ± 1.33 ppm, ranging from a minimum of 1.10 ppm and a maximum of 14.88 ppm.

One-way ANOVA ($p<0.05$) among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients showed a significant difference in the mean value of α-tochopherol ($p<0.0002$ and $R^2=0.32$). Tukey's post-test showed that mean α-tochopherol of CH subjects was significantly different from α-tochopherol of IRIS 1 ($p<0.01$) and IRIS 4 ($p<0.001$). Tukey's post-test showed that mean α-tochopherol of IRIS 3 subjects was significantly different ($p<0.05$) from α-tochopherol of IRIS 4. According to Tukey's post-test, no significant difference was found among IRIS 1, IRIS 2, IRIS 3 and IRIS 4 and among IRIS 2, IRIS 3 and IRIS 4.

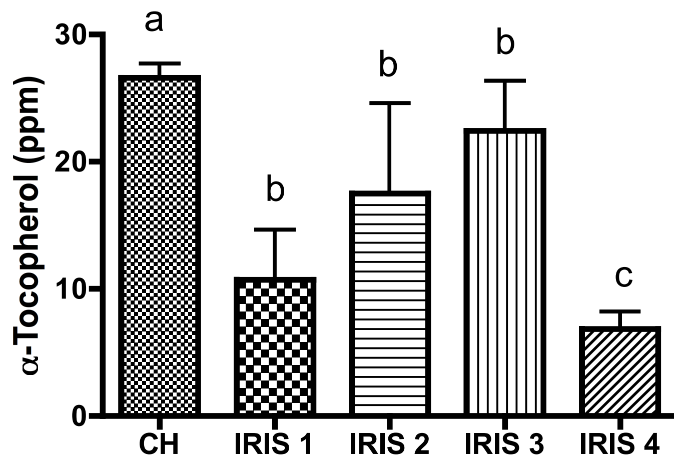

Figura 66: One-way ANOVA analysis of variance of mean α-tochopherol among CH subjects and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients. A significant difference in mean α-tochopherol among different groups was found ($p<0.0002$, $R^2=0.32$). Tukey's post-test showed a significant difference among CH (a), IRIS 1 (b) and IRIS 4 (c) and between IRIS 3 (b) and IRIS 4 (c).

ANOVA test	**α-tochopherol**
One-way analysis of variance	
P value	P<0.0002
P value summary	***
Are means signif. different? (p<0.05)	Yes
Number of groups	5
F	6,627
R^2	0,3207

Tukey's test	**Mean diff**	**q**	**P value**	**95% Cl of diff**
CH vs IRIS 1	15,85	5,399	P < 0.01	4.130 to 27.57
CH vs IRIS 2	9,077	2,699	P > 0.05	-4.350 to 22.50
CH vs IRIS 3	4,139	1,372	P > 0.05	-7.910 to 16.19
CH vs IRIS 4	19,71	6,108	P < 0.001	6.827 to 32.59
IRIS 1 vs IRIS 2	-6,774	1,846	P > 0.05	-21.42 to 7.877
IRIS 1 vs IRIS 3	-11,71	3,490	P > 0.05	-25.11 to 1.687
IRIS 1 vs IRIS 4	3,860	1,089	P > 0.05	-10.29 to 18.01
IRIS 2 vs IRIS 3	-4,938	1,322	P > 0.05	-19.85 to 9.977

IRIS 2 vs IRIS 4	10,63	2,722	P > 0.05	-4.963 to 26.23
IRIS 3 vs IRIS 4	15,57	4,309	P < 0.05	1.144 to 30.00

Pearson correlation analysis ($p<0.05$) between plasma creatinine and α-tochopherol showed a significant correlation in IRIS 2 patients ($p=0.00$, $R^2=0.85$). No significant correlation between plasma creatinine and α-tochopherol was found in CH subjects ($p=0.47$, $R^2=0.02$), in IRIS 1 ($p=0.49$, $R^2=0.04$), in IRIS 3 ($p=0.87$, $R^2=0.00$) and in IRIS 4 ($p=0.94$, $R^2=0.00$) patients.

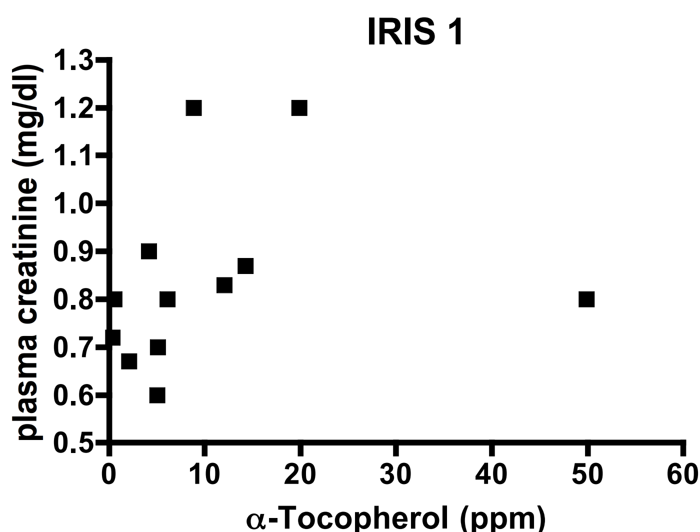

Figura 67: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and α-tochopherol (ppm) in 13 IRIS 1 patients. No significant correlation between plasma creatinine and α-tochopherol was found ($p=0.49$ and $R^2=0.04$)

PEARSON CORRELATION ANALYSIS	**α-tochopherol**
Number of XY Pairs	13
Pearson r	0,2191
95% confidence interval	-0.4060 to 0.7045
P value (two-tailed)	0,4939
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,04800

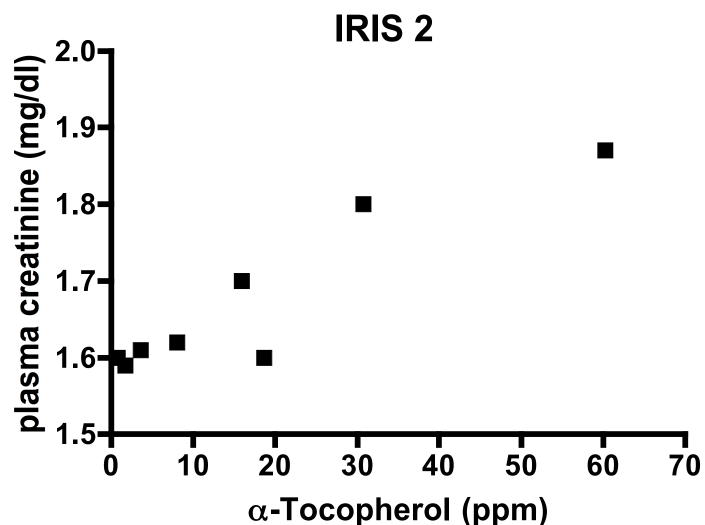

Figura 68: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and α-tochopherol (ppm) in 7 IRIS 2 patients. A significant correlation between plasma creatinine and α-tochopherol was found ($p=0.00$ and $R^2=0.85$)

PEARSON CORRELATION ANALYSIS	**α-tochopherol**
Number of XY Pairs	7
Pearson r	0,9245
95% confidence interval	0.6306 to 0.9865
P value (two-tailed)	0,0010
P value summary	**
Is the correlation significant? (alpha=0.05)	Yes
R^2	0,8547

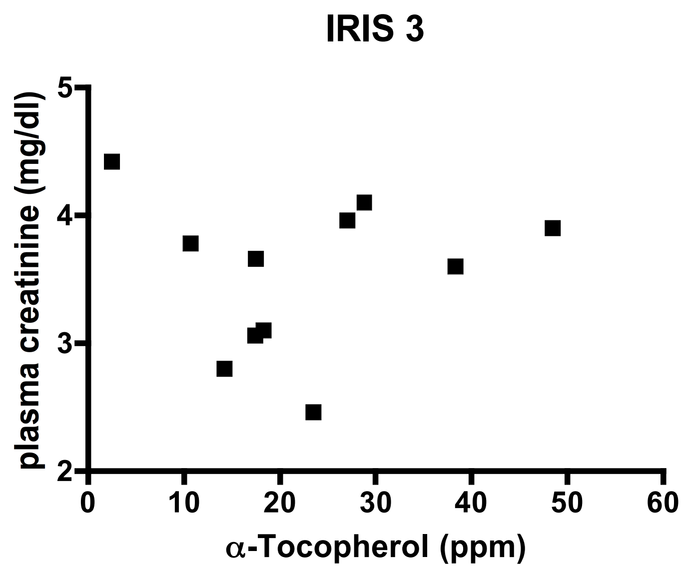

Figura 69: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and α-tochopherol (ppm) in 13 IRIS 3 patients. No significant correlation between plasma creatinine and α-tochopherol was found ($p=0.87$ and $R^2=0.00$)

PEARSON CORRELATION ANALYSIS	**α-tochopherol**
Number of XY Pairs	13
Pearson r	0,05218
95% confidence interval	-0.5655 to 0.6324
P value (two-tailed)	0,8789
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,002723

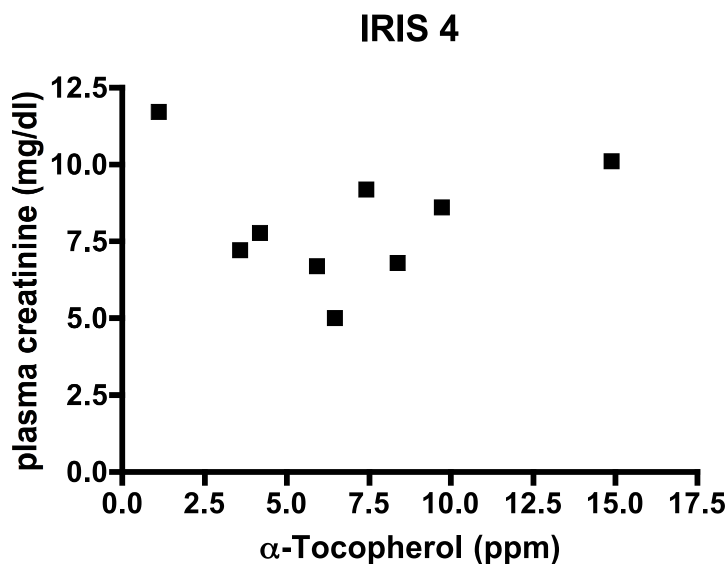

Figura 70: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and α-tochopherol (ppm) in 11 IRIS 4 patients. No significant correlation between plasma creatinine and α-tochopherol was found ($p=0.94$ and $R^2=0.00$)

PEARSON CORRELATION ANALYSIS	**α-tochopherol**
Number of XY Pairs	11
Pearson r	0,02605
95% confidence interval	-0.6494 to 0.6785
P value (two-tailed)	0,9470
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,0006784

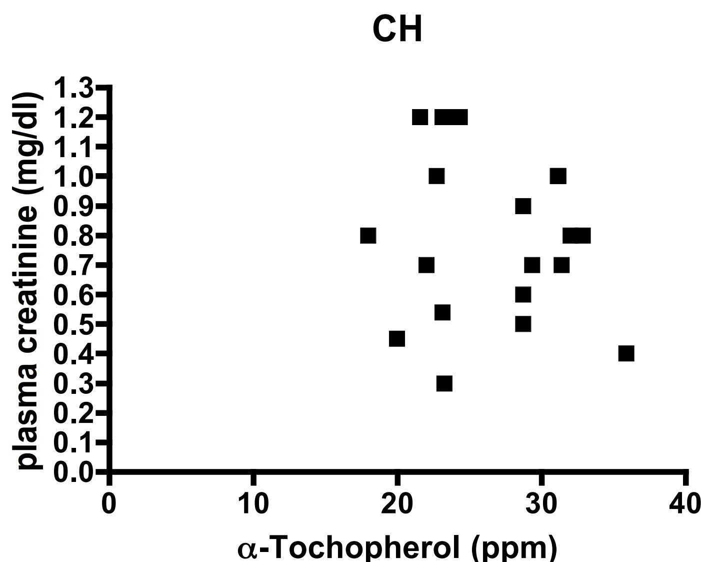

Figura 71: Pearson correlation analysis ($p<0.05$) between plasma creatinine (mg/dl) and α-tochopherol (ppm) in 20 CH subjects. No significant correlation between plasma creatinine and α-tochopherol was found ($p=0.47$ and $R^2=0.02$)

PEARSON CORRELATION ANALYSIS	**α-tochopherol**
Number of XY Pairs	20
Pearson r	-0,1712
95% confidence interval	-0.5706 to 0.2937
P value (two-tailed)	0,4705
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R^2	0,02931

Contingency analysis ($p<0.05$), through χ^2 test, between the number of subjects with α-tochopherol above and below 21.6 (ppm) in CH subjects and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients showed a significant difference ($p=0.0001$). In CH group 3 subjects showed α-tochopherol below 21.6 ppm, while 17 subjects α-tochopherol above 21.6 ppm. In IRIS 1 group, 12 patients showed α-tochopherol below 21.6 ppm, while only 1 patient showed α-tochopherol above 21.6 ppm. In IRIS 2 group, 5 patients showed α-tochopherol below 21.6 ppm, while 2 patients showed α-tochopherol above 21.6 ppm. In IRIS 3 group, 7 patients showed α-tochopherol below 21.6 ppm, while 6 patients showed α-tochopherol above 21.6 ppm. In IRIS 4 group, all 11 patients showed α-tochopherol below 21.6 ppm.

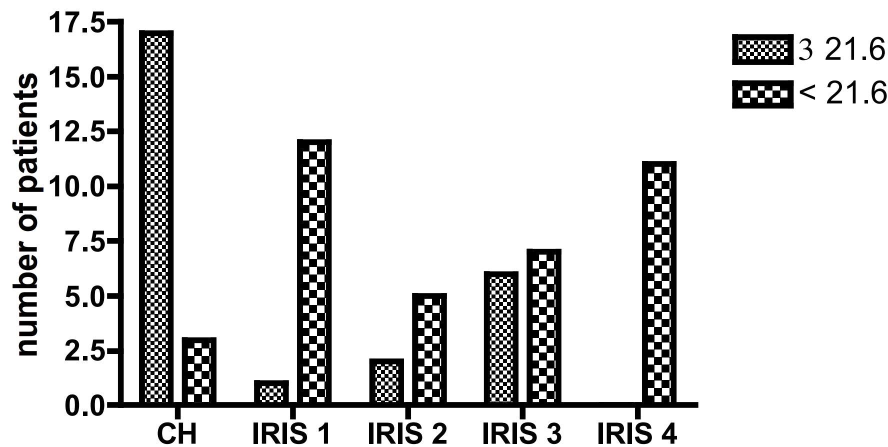

Figura 72: contingency analysis (p<0.05) between the number of subject with α-tochopherol below and above 21.6 ppm in CH and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients, showed a significant difference (p=0.0001)

χ^2 TEST	α-TOCHOPHEROL
Chi-square, df	30.29, 4
P value	P<0.0001
P value summary	***
One- or two-sided	NA
Statistically significant? (alpha<0.05)	Yes

Contingency analysis (p<0.05), through χ^2 test, between the number of survived subjects and dead subjects in the group of CKD patients with α-tochopherol above and below 21.6 ppm showed no significant difference. In the group of subjects with α-tochopherol below 21.6 ppm, 14 dogs survived, while 18 died after a period of time of 455 days. In the group of subjects with α-tochopherol above 21.6 ppm, 5 dogs survived, while 6 died after a period of time of 455 days.

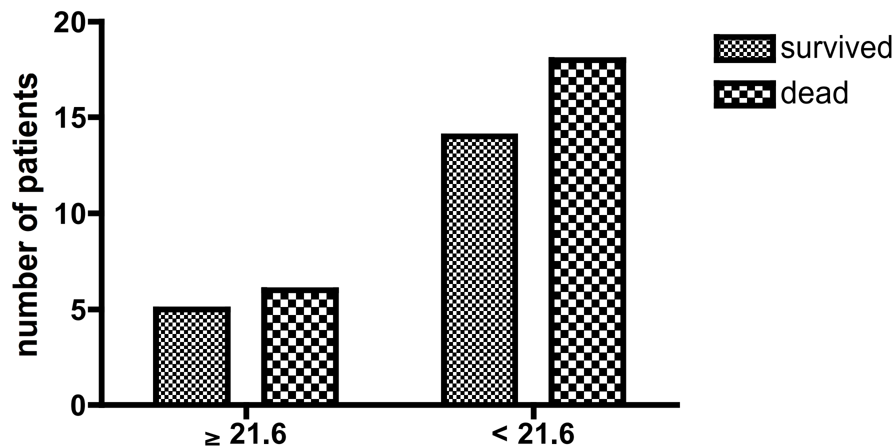

Figura 73: contingency analysis, through χ^2 test, of survived and dead CKD patients in the group of dogs with α-tochopherol below and above 21.6 ppm. No significant difference between the groups of subjects was found. In group with α-tochopherol below 21.6 ppm, 14 dogs survived, while 18 died. In group with α-tochopherol above 21.6 ppm, 5 dogs survived, while 6 died.

χ^2 TEST	α-TOCHOPHEROL
Chi-square, df	0.009644, 1
P value	0,9218
P value summary	ns
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	No

Log-rank test ($p<0.05$) between the group of subjects with α-tochopherol below 21.6 ppm and the group of subjects with α-tochopherol above 21.6 ppm showed no significant difference. In the group of patients with α-tochopherol below 21.6 ppm, 14 dogs survived and 18 died in a 455 day period, while in the group of patients with α-tochopherol above 21.6 ppm, 5 dogs survived while 6 died. Kaplan-Meier survival curve showed no significant difference in the percentage of survival of CKD patients with α-tochopherol below 21.6 ppm compared to patients with α-tochopherol above 21.6 ppm.

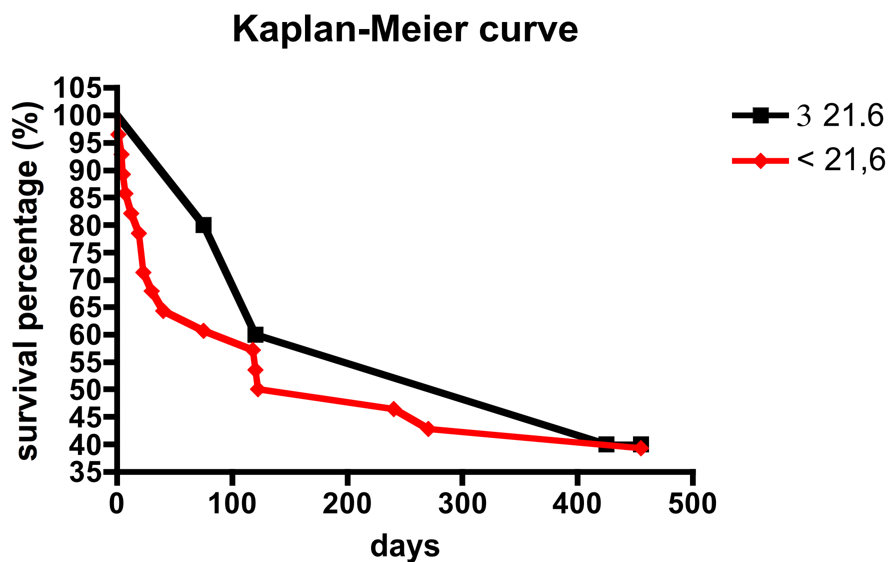

Figura 74: Kaplan-Meier survival curve between the group of CKD patients with α-tochopherol below 21.6 ppm and the group of patients with α-tochopherol above 21.6 ppm. No significant difference in percentage of survival between the two groups of patients was found.

CHAPTER 13: DISCUSSION

In canine and feline population as well as in humans, CKD plays an important epidemiological role, showing an even increasing prevalence. Although different definitions are present, CKD refers to a chronic and progressive clinical syndrome, which inexorably moves to ESRD and uraemia. Since the seventies, veterinary haemodialysis and renal transplantation became a reality in the USA and, at present, these techniques are becoming available all over the world. Anyway, the high costs of supplies and materials and the reticence of some owners and veterinarians towards these very high tech procedures, have been limiting their use to a few number of cases. It is also important to notice that, even in human medicine, most recent guidelines suggest to manage CKD patients with diet and medical therapy as longer as possible before using haemodialysis or continuous renal replacement therapy (CRRT). As a consequence, the importance of early diagnosis has grown and new methods have been developed in order to identify CKD as soon as possible. The recent development of a renal standardization group (International Renal Interst Society – IRIS) led to the formulation of diagnostic guidelines, which should help private practitioners in diagnosing and treating CKD. Both feline and canine CKD patients are classified, according to plasma creatinine, in four stages of severity. Although IRIS stage 2, 3 and 4 are characterized by high plasma values of creatinine, IRIS stage 1 encloses asymptomatic patients with plasma creatinine below 1.4 mg/dl. At present, most private practitioners base diagnosis of CKD on plasma creatinine and urea. These two parameters have the advantage to be extremely cheap and easy to determine, but they have demonstrated late and inaccurate in diagnosing CKD. Plasma creatinine can be use to diagnose IRIS 2, 3 and 4 patients but it is completely ineffective for earlier stages. Furthermore, as patients with plasma creatinine above the reference range have already lost more than 70% of total renal function, IRIS 2, 3 and 4 represent advanced stages of the disease. So the aim of early diagnosis should be the identification of IRIS 1. GFR remains the only test capable to identify IRIS 1 patients. In fact, this group of patients is often asymptomatic and characterized by plasma creatinine within the normal range. As UP/UC and systolic blood pressure are risk factors, which cannot diagnose CKD by themselves, IRIS 1 patients can be detected only through GFR. The GFR

determination method in use at the Veterinary Teaching Hospital "Mario Modenato" uses a five-sample system (5', 15', 60', 90' and 180'), based on the plasma clearance of iohexol, which was developed in conjunction with the Section of Pharmacology and Toxicology of our Department. This simplified method was obtained from a 24 hour reference scheme and it was validated both in healthy and in CKD canine patients. The HPLC detection system showed very accurate and simple to be performed. The low number of blood samples and time required, compared to other plasma clearance methods, significantly increase the compliance of both owners and patients. The introduction of GFR test in nephrological consultations to assess the effective functional renal mass of patients, led to the identification of a growing number of IRIS 1 subjects, which, otherwise would have been undiagnosed. Together with early diagnosis of CKD, a deep interest towards prognostic factors developed. In human medicine several prognostic factors of CKD have been recognized and studied, in order to monitor and predict the progression of the disease. Most factors, in fact, show both a prognostic and progressive nature. Modifications of these factors were associated with a faster progression of CKD and entry into dialysis. Although the role of these markers in the progression of CKD has been widely demonstrated in human medicine, the mechanisms by which they act are still being studied. Particularly, CKD progression has been associated with alterations in calcium-phosphate metabolism, cardiovascular injury, inflammation and oxidative distress. The present study took into consideration one marker for each of these pathological conditions, assessing its concentration in different IRIS stages and in clinically healthy subjects to evaluate its potential prognostic role.

Ionized calcium, total calcium, calcium corrected for albumin (cCaAlb), calcium corrected for total proteins (cCaPt)

As universally known, homeostasis of calcium is primarily regulated by the activity of PTH, calcitonin and vitamin D, so parathyroid glands, bones, intestine and kidneys strictly interact to maintain physiologic concentration of calcium. As a consequence, any pathological condition involving one or more of these organs can deeply affect calcium homeostasis[1]. The importance of mineral metabolism and calcium-phosphate status in human CKD patients was documented by an intense scientific, epidemiologic and clinical research, which recently led

the Kidney Disease Improving Global Outcomes (KDIGO) group to the formulation of a new classification, named Chronic Kidney Disease – Bone and Mineral Disorder (CKD-BMD)[2]. In veterinary medicine, available evidence in spontaneous CKD cats and in experimental CKD dogs reported that abnormalities in calcium and phosphate metabolism show a negative effect on residual kidney function and survival. In cats, for each 1 mg/dl increase in serum phosphate there is an 11.8% increase in the risk of mortality[3,4,5]. Ionized calcium, total calcium, calcium corrected for albumin (cCaAlb) and calcium corrected for total proteins (cCaPt) were assessed in all 301 CKD canine patients presented to the Veterinary Teaching Hospital "Mario Modenato" in a three year period for nephrologic consultation and in 125 clinically healthy (CH) subjects. Ionized calcium was considered the gold standard and two correction equations, one based on serum albumin and one based on total proteins, were taken into consideration. These equations derived from human medicine and were introduced in veterinary medicine because of the difficulties many veterinarians have in determining ionized calcium. The aim of the present study was to assess calcemic status in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients and in CH subjects and to test the accuracy of total calcium, cCaAlb and cCaPt in predicting ionized calcium. Present results (ANOVA; $p<0.05$) showed that mean concentration of ionized calcium in CH and in the four stages of CKD was significantly different ($p<0.0001$, $R^2=0.14$). In particular, IRIS 3 and IRIS 4 patients showed a mean ionized calcium concentration significantly lower, compared to CH subjects and IRIS 1 and IRIS 2. IRIS 4 patients showed a mean ionized calcium concentration below the reference range (1.06 ± 0.03 mmole/l). Furthermore, contingency analysis reported that the number of hypocalcemic patients significantly ($p<0.0001$) increased with the severity of the disease. These data were in partial agreement with some previous works[6,7] on CKD dogs, which reported ionized calcium within the reference range or slightly decreased. Anyway, these works considered a lower number of patients and did not identify IRIS 1 subjects by GFR but by persistent renal proteinuria. Despite what previously found, one-way ANOVA analysis showed that, although mean ionized calcium concentration between CH and CKD was significantly different, significance increased dramatically in IRIS 3 and 4. This finding may be explained by both the onset of metabolic acidosis and secondary hyperparathyroidism (HPTH). Metabolic acidosis is a very common condition in CKD due to the lower renal excretion of acid radicals, phosphate and sulphate compounds, as well as reduced renal

absorption of bicarbonate and low ammoniagenesis. Calcium, in fact, binds to the albumin carboxyl group, that represents the same site at which H^+ usually binds. As, during metabolic acidosis, H^+ radicals do not dissociate easily from carboxyl groups, calcium has less opportunities to bind albumin, resulting in an increase in serum concentration[8]. Higher concentrations of ionized calcium were reported in induced acidosis dogs and calves[9,10]. Moreover, metabolic acidosis itself can cause calcium disturbances characterized by urinary calcium loss, increase bone calcium absorption and deficient synthesis of vitamin D[8]. On the other hand, the significant increase in the number of hypocalcemic patients in IRIS 3 and 4, may be determined by HPTH. High serum phosphate levels inhibit the activity of renal 1-α-hydroxylase, causing a reduction in vitamin D and ionized calcium concentration. This condition stimulates PTH release, which diminishes tubular phosphate absorption and increases phosphaturia. Unfortunately, the restore of normal serum levels of vitamin D and ionized calcium is only a transitory phenomena[7]. So, after a first rebalance of calcemia, CKD patients are doomed to develop hypocalcemia. The increasing prevalence of hypocalcemia with the progression of CKD underlined the importance of measuring ionized calcium, especially in IRIS 3 and IRIS 4 patients. The early detection of a hypocalcemic status can help practitioners in treating patients and avoiding clinical symptoms. Anyway, the assessment of ionized calcium can be sometimes difficult in a routine clinical setting, where calcemic status is often determined by measuring total or corrected calcium. In our study Pearson correlation analysis between ionized calcium and total calcium, ionized calcium and cCaAlb, ionized calcium and cCaPt, showed no significant correlation in IRIS 1 patients. The lack of correlation between the gold standard and each of the methods used for predicting ionized calcium, underlined the low accuracy of these parameters in a very early stage of the disease. As CKD patients can remain in an IRIS 1 stage for a long period of time (from months to years), the use of ionized calcium in this group of subjects can show significantly important for an accurate assessment of calcemic status. Being able to discover calcium disorders in an early phase of CKD can be of fundamental importance, in order to establish therapeutic and dietary modifications, which prevent the onset of HPTH and the progression of the disease. In agreement with previously reports, both total and corrected (cCaAlb, cCaPt) calcium showed inaccurate in predicting ionized calcium. This observation may find explanation in the fact that both analyzed equations are based only on serum albumin and

total proteins, avoiding to consider other compounds like citrate, phosphate and solphate. Calcium may, in fact, form complexes with bicarbonate, lactate, citrate, phosphate and oxalate, determining significant modifications in complexed fraction, which cannot be discovered by correcting total calcium for albumin or total proteins. Furthermore, alterations of normal distribution of calcium into the three serum fractions may occur without causing modifications of total calcium. As a consequence the only assessment of serum total calcium may result perfectly normal, despite the presence of calcium homeostasis disorders. A significant correlation was found both in IRIS 2 and IRIS 3 patients. In IRIS 2, cCaAlb (Pearson r=0.52) and cCaPt (Pearson r=0.51) showed a better correlation with ionized calcium, compared to total calcium (Pearson r=0.42). On the contrary, in IRIS 3 total calcium showed the best correlation (Pearson r=0.68) with ionized calcium, compared to cCaAlb (Pearson r=0.49) and cCaPt (Pearson r=0.52). The poorer correlation ability of cCaAlb and cCaPt in IRIS 3, compared to IRIS 2, may be due to a higher prevalence of clinical symptoms at this stage of the disease. IRIS 3 patients are often very symptomatic and may present albumin and serum protein disorders, which can significantly affect the accuracy of cCaAlb and cCaPt. Vomiting, malnutrition, diarrhea and proteinuria can be responsible for intense protein loss and hypoalbuminaemia. The worsening of clinical status, together with the inexorably progression of the disease may be responsible for the lack of correlation between ionized calcium and cCaAlb and ionized calcium and CCaPt in IRIS 4. IRIS 4 patients, in fact, showed a significant correlation only between ionized and total calcium, while no correlation was present between ionized and cCaAlb and ionized and cCaPt. At this stage of CKD, the measurement of ionized calcium becomes essential for an accurate assessment of calcemic status. The wide fluctuations in serum albumin and total proteins, due to the worsening of symptoms, and the high prevalence of hypocalcemia, compared to other stages, should be a valid reason to avoid the use of total or corrected calcium.

Ca x P product (Ca x P)

In human medicine, increasing evidences suggested a strict relationship between cardiovascular mortality risk and hyperphosphatemia in CKD. Cardiovascular disease represents, in fact, the leading cause of death in the majority of CKD subjects, not only before

and on dialysis program, but also after successful renal transplantation. An independent association between serum phosphate and mortality, as well as between serum phosphate and progression of CKD, has been widely demonstrated by numerous studies. For this association different mechanisms were supposed. First of all, elevated phosphate levels may directly induce vascular injury by stimulating vascular calcification, enhancing mineral deposition in the vascular wall and promoting expression of bone-forming transcription factors and proteins[11]. Hyperphosphatemia seems to be associated with increasing myocardial hypertrophy, cardiac fibrosis and arterial wall thickening, while high PTH values causes intracellular calcium overload and impaired myocardial energy production. Secondly, high serum phosphate may increase the production of reactive oxygen species, as previously documented in cows[12]. Another consequence of hyperphosphatemia is represented by secondary renal HPTH. The onset of HPTH predisposes CKD patients to renal osteodistrophy and to the toxic effects of PTH, as uremic toxin. The increased mortality risk in CKD subjects, showing high levels of serum phosphate, may be determined by an elevated Ca x P product (Ca x P). In humans, Ca x P levels greater than 70 mg/dl were associated with a significantly higher relative risk of death. This data well correlated with previously reports, in which Ca x P above 60 and 70 mg/dl was strictly related to high risk of metastatic calcifications. Myocardial and pulmonary calcium content in CKD patients with Ca x P above 70 was markedly higher, as well as the prevalence of valvular calcifications. Arterial calcifications, in fact, were linked to elevated Ca x P levels and a coronary artery calcium score 2.5 to 5 times higher was found in dialysis patients, compared to non-dialysis ones[13]. Elevated serum phosphate and Ca x P values were both demonstrated significant predictors of cardiovascular mortality in CKD human patients. For this reason, actual guidelines recommend to maintain phosphate and Ca x P between 3.0 and 5.0 mg/dl and below 55 mg/dl respectively[14]. The aim of the present study was to assess Ca x P in CH and in IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients and to evaluate its relationship towards mortality. In CH group all 125 patients showed Ca x P values above 70 mg/dl, with a mean value (30.22 ± 0.85 SE) significantly ($p<0.0001$) different from CKD. The mean Ca x P level showed a progressive increase of its serum concentration, according to the progression of the disease, even if, average Ca x P above the normal value, was found only in late stages. Although no significant difference was found, IRIS 1 and IRIS 2, in fact, showed a mean serum Ca x P of 39.06 ±

3.03 SE and 34.48 ± 4.73 SE respectively. It is to be noticed that no-one of IRIS 1 patients was under low-phosphate diet or therapy, while in IRIS 2 some dogs have already started renal diet. This data may be responsible for the higher mean value of Ca x P of IRIS 1 compared to IRIS 2. Most IRIS 2 patients were, in fact, referred from private practitioners, who had already started a renal diet. In IRIS 3 and IRIS 4 patients, instead, mean Ca x P was significantly higher than the cut off value of 70 mg/dl. Patients of IRIS 3 and IRIS 4 stage showed an average Ca x P of 77.92 ± 10.17 SE and 195.80 ± 126.20 SE respectively. ANOVA and Tukey's post-test identified a significant difference not only among IRIS 3, IRIS 4, other stages and CH, but also between IRIS 3 and IRIS 4. Mean Ca x P level showed the highest value in IRIS 4 group, where it reached a value more than twice the risk level. According to present data and to the fact that the number of hypocalcemic patients increases with the progression of the disease, phosphate accumulation and Ca x P alterations seemed to develop more in late rather than early stage of CKD. Although a significant increment in Ca x P was found according to CKD progression, Pearson test showed no significant correlation towards plasma creatinine in anyone of IRIS stages and in CH group. As previously reported for hypocalcemia, also Ca x P increase seemed to be independent from plasma creatinine modifications and increments in Ca x P concentration did not correspond to the same increment in plasma creatinine. A significant difference in the number of patients with Ca x P below and above 70 mg/dl was found according to the progression of CKD. The number of subjects with Ca x P above 70 mg/dl increased significantly ($p<0.0001$) with IRIS progression. In particular, all patients of IRIS 4 presented Ca x P above reference value. The same significant difference ($p<0.0008$) between the number of survived and dead patients in Ca x P group above and below 70 mg/dl was found. The number of dead patients over a 455 day period was, in fact, significantly higher in Ca x P over 70 group. This finding was in complete agreement with previous human reports, which underlined the elevated prognostic value of Ca x P. High serum phosphate and Ca x P have been demonstrated to be correlated to increased mortality risk and, in renal transplant patients, they represented additional and independent risk factors. In the present study, an even more important confirmation of the key role of Ca x P in predicting progression and mortality of CKD, derived from log rank analysis between the group of subjects with Ca x P below and above 70 mg/dl. Kaplan-Meier curves of the two groups of patients were, in fact, significantly different ($p<0.0002$) from each other.

CKD patients with Ca x P above 70 mg/dl showed a percentage of survival significantly lower, compared to the other group. This finding may suggest that in dogs, as well as in humans, the prognostic relevance of Ca x P in predicting mortality. According to present data, alterations of phosphate metabolism and subsequent increase in Ca x P seemed to be more characteristic of the late stages of renal disease. Anyway, the significantly lower percentage of survival of patients with Ca x P above 70 mg/dl resulted independent from the IRIS stage, which the patient belonged to. Early detection of phosphate alterations and Ca x P modifications should be taken into consideration, in order to identify patients at higher risk of progression of CKD. Early recognition of these high risk patients may help veterinarians in correcting calcium-phosphate alterations and in reducing mortality rate.

Cardiac troponin I (cTnI)

A significant relationship between CKD and an increased risk of cardiovascular disease has been widely demonstrated in human medicine. The risk of cardiovascular mortality is particularly high in the late stages of CKD and in end stage renal disease (ESRD). These patients, in fact, showed an increased prevalence of vascular and valvular calcifications, arterial stiffness and cardiac fibrosis, which may predispose patients to inadequate coronary reserve and cardiac ischemia. Cardiovascular complications represent a very frequent condition in CKD, which show different pathogenetic mechanisms[1,2]. Cardiac diseases account for about 45% of all deaths in ESRD patients and, in haemodialysis subjects, about 20% of cardiac deaths are attributed to acute myocardial infarction[3]. Identification of such mechanisms and risk factors may be of dramatic importance in assessing therapy and preserving both cardiac and renal function. In human medicine, different cardiac biomarkers were tested and, among them, cardiac troponins T and I had a very important position. Both cardiac troponins are frequently elevated in the absence of acute coronary syndrome in patients with various degrees of kidney disease. cTnT showed a more frequent increase in asymptomatic ESRD patients, compared to cTnI, with a reported prevalence between 30% and 80%. In the same class of patients, cTnI reported a percentage of increase between 5% and 18%. The prevalence of increase of cTnC and cTnI showed higher values with the progression of CKD. Anyway, despite of a lower prevalence of increase in ESRD, cTnI

showed a very good prognostic marker of cardiovascular complications, due to lower incidence of elevation and expression in non-cardiac tissues[1]. At present, cTnI is considered the gold standard measurement to detect myocardial injury and to predict mortality and future cardiac events. cTnI serum concentration starts to rise within 2 hours after myocardial damage, reaching a peak at 12 – 24 hours. Furthermore, in acute coronary occlusion canine models cTnI concentration correlated with infarct size, suggesting a potential role as marker of the extent of myocardial damage. Shorter survival times were also found in dogs with acquired cardiac disease and cTnI above normal values[4]. Recently reports in veterinary medicine, demonstrated that CKD dogs and cats often present cTnI above physiologic levels. The percentage of increase was a little more elevated in dogs (78%) than in cats (72%). Patients with myocardial injury showed high values of cTnI, even if obvious ante-mortem cardiac involvement was not present. cTnI appeared, in fact, a highly specific marker of myocardial damage but not a specific clinical marker of primary cardiac disease or heart failure. Moreover mild subclinical heart disease did not result in marked elevation of cTnI and hypertension did not correlate with cTnI increase. Although a relationship between high levels of cTnI and myocardial injury was demonstrated, the mechanisms of cTnI elevation in CKD patients are still unknown. In human patients the most accredited hypothesis of cTnI increase in CKD subjects referred to independent and un-associated co-morbidity of coronary artery disease. However, because coronary artery disease is not a feature of veterinary patients, this hypothesis could not explain the mechanism in companion animals[5]. In veterinary CKD patients, as well as in humans, cTnI elevation appeared more frequent in the late stages rather than in early ones. As no data regarding IRIS classification and the survival of patients are available, the present study analyzed cTnI concentration in patients belonging to all IRIS classes, the correlation towards plasma creatinine and the survival percentage in subjects with cTnI above and below the reference value. Considering a physiologic value of cTnI of 0.2 ng/ml, the present results showed that all CH subjects had mean cTnI level below 0.2 ng/ml. On the contrary, CKD patients, belonging to different classes of severity, reported a mean value of cTnI above 0.2 ng/ml. Mean cTnI tended to increase according to the progression of the disease, showing a value of 0.98 ± 0.28 ng/ml in IRIS 1, 6.90 ± 6.36 ng/ml in IRIS 2, 6.19 ± 3.99 ng/ml in IRIS 3 and 11.80 ± 5.15 ng/ml in IRIS 4. One-way ANOVA ($p<0.05$) of these data showed a significant difference ($p<0.02$) among mean cTnI values of

CH, IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients. Although a significant difference was present, Tukey's post-test reported no significant difference among CH, IRIS 1, IRIS 2 and IRIS 3 and among IRIS 1, IRIS 2, IRIS 3 and IRIS 4. The only significant difference was between CH and IRIS 4 ($p<0.05$). These findings were in agreement with previous studies in human and veterinary medicine, in which the degree of cTnI augmented with the progression of the disease. It is to be noticed that, despite some patients presented cTnI below reference, mean cTnI concentration was above 0.2 ng/ml even in early, asymptomatic IRIS 1 subjects. Contingency analysis ($p<0.05$), as well, showed a significant ($p<0.0001$) increase in the number of subjects with cTnI above 0.2 ng/ml according to CKD progression. Although the significant tendency of cTnI towards increasing mean values with the progression of CKD, Pearson test reported no significant correlation between cTnI and plasma creatinine in no one of IRIS stages. No relationship between cTnI and plasma creatinine was found. As reported for Ca x P, the link between cTnI elevation and CKD progression seemed to be independent from plasma creatinine. According to mortality analysis, contingency analysis ($p<0.05$) regarding the number of survived and dead patients between the group of subjects with cTnI below and above 0.2 ng/ml, showed a significant difference ($p<0.02$). The number of dead patients was, in fact, significantly higher in the group of subjects with cTnI above 0.2 ng/ml. Similarly, log-rank test ($p<0.05$) showed a significant difference ($p<0.0002$) between the group of patients with cTnI above and below 0.2 ng/ml. CKD subjects with cTnI above 0.2 ng/ml reported a Kaplan-Meier curve of the percentage of survival significantly lower ($p<0.0007$) than the other group. In agreement with what previously reported in human medicine, serum cTnI elevation seemed to be significantly correlated with a higher mortality risk and reduction in percentage of survival. cTnI resulted completely independent from the plasma creatinine value of CKD patients. Furthermore the prognostic role of cTnI did not depend on the severity of the disease (according to IRIS classification) and cTnI appeared an independent risk factor of mortality. Anyway, it is essential to say that the prevalence of cTnI elevation is significantly higher with the progression of IRIS class. As cTnI has been widely demonstrated as a marker of myocardial injury in both animals and humans, the significantly higher prevalence of subjects presenting cTnI above 0.2 ng/ml and mean cTnI above the cut off value with the progression of CKD, may suggest a role of cardiovascular damage in the progression of renal disease. Furthermore, the evidence of patients with cTnI above 0.2 ng/ml

even in IRIS 1 patients and their lower survival percentage over a 455 day period, seemed to indicate that cardiovascular alterations may start even at the very beginning of CKD. Although in human patients no relationship between cTnI increase and obvious ante-mortem cardiac involvement was found, on the basis of present the results, it may be advisable to submit CKD patients with cTnI above 0.2 ng/ml to clinical and instrumental cardiological evaluation.

C-reactive protein (CRP)

In human medicine, C-reactive protein (CRP) was associated with the prevalence, progression and negative outcome of CKD[1]. Inflammation and positive phase proteins strongly predicted outcome in dialysis patients[2]. Increased serum levels of several acute phase proteins, such as CRP, were documented among different patients affected by CKD. However, a direct relationship between inflammatory biomarkers and reduction in GFR was not yet demonstrated[2]. A strong correlation between chronic inflammation and CKD was widely reported in humans and several studies underlined the role of inflammation in promoting morbidity and mortality in such class of patients. In a CKD condition many causes can be responsible for the onset of acute and chronic inflammation. Previous reports identified multiple factors, such as decreasing renal function, uraemia, increased susceptibility to infections and alterations of immune system. Furthermore, additional factors like repeated exposure to dialysis system and subsequent leukocyte activation and production of inflammatory cytokines, were detected in patients undergoing haemodialysis[3]. This class of patients showed a significantly higher risk of inflammation compared to other CKD subjects and many sources of inflammation were identified. The kind of vascular access type was associated with a different mortality risk. Arterio-venous fistula showed the highest infection-induced mortality risk, followed by artero-venous grafts, permanent catheters and temporary catheters. Vascular access inflammation was related both to the initial risk of septicemia and unrecognized infections and biofilm deposited on plastic catheters[4,5]. Finally, haemodialysis patients were found to be at risk of inflammation by possible interactions of circulating monocytes with dialysis membrane or with bacterial products, lipopolysaccharide and bacterial DNA that may be present in dialysate[6]. In patients undergoing chronic

haemodialysis treatment, the repetitive induction of acute phase response seemed to induce a chronic micro-inflammatory state, leading to a variety of long-term complications, including malnutrition and cardiovascular diseases[3]. Inflammation was recognized as a condition affecting prognosis and outcome, not only in haemodialysis patients but also in CKD untreated subjects. Different potential injurious effects of inflammation were reported in human medicine. Firstly, clinical evidences showed hypoalbuminemia a powerful risk factor for mortality and this condition was attributed both to malnutrition and inflammation. Then, the role of hypoalbuminemia, as marker of inflammation, was strongly replaced by CRP. Although both albumin and CRP were demonstrated to be independent predictors of all cause mortality in haemodialysis patients, CRP contributed more to the risk of death. Whatever the cause of inflammation is, an increased synthesis of proinflammatory cytokines and leukocytosis occurs. Inflammation determines various modifications such as reduction in synthesis of negative acute phase proteins (albumin, pre-albumin, transferrin), decreased iron absorption and recycling, increased synthesis of positive acute phase proteins (CRP, SAA, fibrinogen, pentraxin, hepcidin, ceruloplasmin) and generation of a proathergenic lipoprotein profile (low HDL, oxidized LDL, high triglycerides). All these modifications were recognized to be responsible for an increased risk of cardiovascular damage[2]. Augmented atherosclerotic lesions were supposed to be the connection between inflammation and cardiovascular injury. In particular, CRP was thought to be an active factor in atherosclerotic lesions, by binding to damaged cells, with subsequent activation of innate immune system through complement, resulting in foam cells formation. Similarly, CRP is stimulated and attracted by complement release and by induction of pro-inflammatory cytokines. Furthermore, in general population, CRP was demonstrated to be an important risk factor for cardiovascular outcome, like acute myocardial infarction, peripheral vascular disease and all-cause mortality[7]. At present, no data regarding CRP concentration and its relation to mortality risk, are present in canine CKD patients. The present study analyzed CRP concentration in CH subjects and in patients belonging to all IRIS classes, correlation towards plasma creatinine and survival percentage in subjects with CRP above and below the reference value. All CH subjects showed CRP values below reference range and their mean concentration was 1.81 ± 0.36 mg/l. One-way ANOVA ($p<0.05$) reported a significant ($p<0.0001$) difference in mean serum CRP among CH, IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients. IRIS 1 and IRIS 2 patients showed a mean CRP value of

7.48 ± 2.77 mg/l and 4.28 ± 1.27 mg/l respectively. Mean CRP concentration in IRIS 3 and IRIS 4 was above the reference range, with values of 17.92 ± 5.16 mg/l and 20.15 ± 3.32 mg/l respectively. It is interesting to notice that mean serum CRP in late stages of CKD was about twice the reference cut off level. Furthermore, Tukey's post-test underlined that CRP in CH subjects was significantly different ($p<0.001$) from both IRIS 3 and IRIS 4. Similarly, a significant ($p<0.05$) difference was found between mean serum CRP of IRIS 1 and IRIS 4. Instead, no significant difference was present among CH, IRIS 1 and IRIS 2, among IRIS 1, IRIS 2 and IRIS 3 and between IRIS 3 and IRIS 4. In agreement with previously reports in human medicine, in which CRP modifications were found more often in ESRD subjects and in patients undergoing haemodialysis, in dogs CRP alterations seemed to verify more frequently in late stages of CKD, rather than in early phases. These findings seemed to be confirmed even from contingency analysis ($p<0.05$) regarding the number of subjects with CRP above and below 9.7 mg/l in CH and IRIS 1, IRIS 2, IRIS 3 and IRIS 4 dogs. The difference in the number of subjects between the two groups was, in fact, significant ($p<0.0001$), showing a significant increase in the number of subjects with the progression of renal disease. For CRP, as well as for cTnI and Ca x P, Pearson test ($p<0.05$) showed no significant correlation between CRP and plasma creatinine. Once more a significant increase in mean CRP concentration, according to IRIS progression, did not correspond to plasma creatinine changes. These data were in complete agreement with several studies in human medicine, which reported no correlation between CRP elevations and GFR. In humans, CRP was demonstrated an independent and strong predictor of all-cause mortality in haemodialysis patients, showing a very high correlation with mortality. In the present study contingency analysis ($p<0.05$) regarding the number of survived and dead subjects in the group of patients with CRP above and below 9.7 mg/l reported a significant difference ($p<0.0009$). The number of dead patients was, in fact, significantly higher in the group with CRP above 9.7 mg/l, with no regards towards IRIS class. The relationship between CRP and negative outcome of CKD was demonstrated even by log-rank test ($p<0.05$), which showed a significant difference between the two groups ($p<0.0009$). Furthermore, the Kaplan-Meier curve of percentage of survival of the group with CRP above reference level was significantly ($p<0.001$) different from the other group. CKD patients with CRP below 9.7 mg/l showed a significantly higher percentage of survival. According to present data, in canine patients as well as in humans,

CRP seemed to be considered a very good marker of negative outcome and mortality in CKD. The significantly reduced percentage of survival of patients presenting CRP above 9.7 mg/l appeared independent from the IRIS class, which patients belonged to. This finding seemed to underline the independent nature of CRP in predicting negative outcome and may justify its determination even in early phases of the disease.

α-tochopherol

A strict relationship between negative cardiovascular outcome and oxidative stress was widely demonstrated in CKD humans. Particularly, augmented oxidative stress was supposed to be the link between inflammation and cardiovascular cellular damage in CKD patients. This class of subjects, in fact, showed a disequilibrium between pro and antioxidant compounds, which led to a state of increased oxidative stress. Several deficiencies in different components of antioxidant defence barrier, together with an increase in pro-oxidant activity, were found[1]. Oxidative stress was particularly elevated in patients undergoing haemodialysis, rather than in earlier CKD stages and a strong debate, about the relationship between haemodialysis and oxidative stress, is still present. On one hand, membrane bioincopatibility and endotoxin challenge, related to dialysis, seemed to worsen oxidative stress. On the other, haemodialysis sessions may replace some antioxidant properties[2]. Anyway it was demonstrated that even a single haemodialysis treatment is able to increase lipid peroxides and decrease antioxidants[3]. Furthermore, haemodialysis patients showed an augmented incidence of coronary artery disease and mortality, when compared to general population[4]. Atherosclerotic injuries were recognized as the leading cause of cardiovascular alterations in ESRD patients and atherosclerotic process seemed to be increased by high oxidative stress[5]. This finding correlated with the acceleration of atherosclerotic lesion onset in diabetic and uremic subjects[1]. Up to 72% of uremic patients, in fact, reported increased intima-media thickness of the carotid and femoral arteries, by the presence of atherosclerotic plaques[6]. Oxidized forms of low-density lipoproteins (LDL) were demonstrated to be deeply involved in atherogenetic process and high serum concentrations were found in haemodialysis subjects. Among different compounds of the antioxidant barrier, α-tochopherol showed an inhibiting action towards LDL oxidation[1]. In CKD patients, elevated serum concentrations of urea-

derived cyanate are responsible for carbamylation of protein components of LDL. Carbamylation of LDL was recognized as a non-traditional risk factor for the development of cardiovascular diseases. In such a condition, endothelial cells attract monocytes and ingest carbamylated LDL, forming foam cells. This process leads to the formation of atherosclerotic plaques, which are composed by macrophages and foam cells, covered by a fibrous cap. A significant in vitro effect of α-tochopherol against LDL carbamylation was recently demonstrated[7]. α-tochopherol represents an effective, lipid-soluble, antioxidant of biological membrane of cells. Among its functions, α-tochopherol shows a protective action of cellular structures towards damages caused by oxygen free radicals and reactive products of lipid peroxidation. α-tochopherol is not only a powerful antioxidant, but it also contributes to the regulation of metabolic activity and cell proliferation and death of various cellular types, such as smooth muscle cells, endothelial cells and leukocytes[8]. Several studies reported that, although α-tochopherol levels were decreased in long-term haemodialysis patients compared to general population, this finding was unrelated to the patients' prognosis. Previous human reports showed that α-tochopherol integration was not able to reduce mortality and, in same cases, may also act as a pro-oxidant agent[9]. Furthermore, α-tochopherol level was a mortality risk factor only linked to other factors, such as nutritional parameters[10]. On the other hand, other studies reported a protective effect of α-tochopherol to prevent renal oxidative damage. The onset of antioxidant renal effect seemed to be related and dependent on dose and duration of treatment[11]. In human CKD patients, infact, a 14-week oral supplementation of α-tochopherol at a dosage of 600 mg/day resulted effective[8]. In veterinary medicine, the dietary supplementation of 200 IU/kg of α-tochopherol was found to reverse established glomerulosclerosis and tubulo-interstitial injury in CKD rats[12] and was demonstrated an effective antioxidant in different animal species. A significant antioxidant activity of an association of α-tochopherol, vitamin C and β-carotene was reported in CKD cats[13]. As no data are currently available about α-tochopherol concentration in CKD canine patients belonging to different IRIS stages, the present study analyzed the serum concentration of α-tochopherol in CH and CKD patients at various degree of severity. Furthermore, correlation analysis towards plasma creatinine and mortality in the group of patients with α-tochopherol levels above and below reference was assessed. In CH subjects mean α-tochopherol value

was above the reference value (26.56 ± 1.11 ppm), while in IRIS 1 and IRIS 2 patients mean serum value was 10.71 ± 3.93 ppm and 17.48 ± 7.07 ppm respectively. IRIS 4 patients, instead, showed a mean value above reference range 22.42 ± 3.88 ppm. One-way ANOVA ($p<0.05$) among CH, IRIS 1, IRIS 2, IRIS 3 and IRIS 4 showed a significant difference ($p<0.0002$) and Tukey's post-test identified a significant difference between mean α-tochopherol of CH and IRIS 1 ($p<0.01$) and between CH and IRIS 4 ($p<0.001$). Moreover, mean α-tochopherol of IRIS 3 was significantly ($p<0.05$) different from IRIS 4. Instead, no significant difference was found among IRIS 1, IRIS 2, IRIS 3 and IRIS 4 and among IRIS 2, IRIS 3 and IRIS 4. Interestingly, the highest mean value of α-tochopherol were found in IRIS 1 and in IRIS 4, while IRIS 2 and 3 showed a significantly lower concentration. On the basis of human data, it is difficult to interpreter present results, because significantly reduced concentrations of α-tochopherol were mainly reported in ESRD and haemodialysis patients. It can be hypothesized that the finding of lower α-tochopherol concentrations in very early and late stages of CKD may be due to a more intense consumption of antioxidant compounds in these phases. So, it would be extremely interesting to measure even reactive oxygen metabolite production, in order to perform a correlation analysis with the concentration of α-tochopherol. According to Pearson correlation analysis ($p<0.05$), no significant correlation was found between serum α-tochopherol and plasma creatinine, with the exception of IRIS 2 ($p<0.00$). In IRIS 2 patients, in fact, α-tochopherol seemed to reduce according to the increase in plasma creatinine. It is to be said that the low number of patients enrolled in IRIS 2 may have influenced results. Further studies should be necessary. Contingency analysis ($p<0.05$) referring to the number of subjects with α-tochopherol below and above 21.6 ppm among CH, IRIS 1, IRIS 2, IRIS 3 and IRIS 4 patients, showed a significant difference ($p<0.0001$). The number of patients with α-tochopherol above the reference significantly reduced with progression of IRIS stage and in IRIS 4 no patients presented α-tochopherol above 21.6 ppm. In agreement with previous studies conducted in human medicine, α-tochopherol concentration significantly decreased with the progression of CKD. In the present study, contingency analysis ($p<0.05$) regarding the number of survived and dead subjects in the group of patients with α-tochopherol below and above 21.6 ppm showed no significant difference. Similarly, both log-rank test ($p<0.05$) and Kaplan-Meier curve showed no

significant difference in the percentage of survival between dogs with α-tochopherol below and above 21.6 ppm. Although a significant decrease in α-tochopherol was found according to progression of CKD, no relationship towards mortality risk was found. In agreement with several human studies, α-tochopherol did not seem a good marker of negative outcome. Anyway, the significant reduction in its serum concentration with CKD progression, should let think about an oral integration of α-tochopherol, in order to reduce renal oxidative damage.

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