Original Article

Investigation of hallmarks of carbonyl stress and formation of end-products in feline Chronic Kidney Disease as markers of uremic toxins

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Abstract

Objectives

Cats are commonly affected by chronic kidney disease (CKD). Many of the reactive carbonyl intermediates and end-products originating from the oxidative stress pathways are recognized as uremic toxins and may play a role in the progression of chronic renal failure. The aim of the present study is to confirm whether carbonyl stress and end product formation are higher in cats affected by CKD than in healthy cats and to assess whether angiotensin-converting-enzyme (ACE) inhibitors might affect these hallmarks.

Methods

Twenty-two cats were matched according to age and body condition score (BSC) and divided into three groups: control group (CG; n = 6), cats with chronic kidney disease (CKD; n = 11) and cats with chronic kidney disease treated with angiotensin-converting-enzyme (ACE) inhibitors (ACE; n = 5). Serum analysis was carried out to measure the levels of Pentosidine, Carboxymethyllysine, Advanced Oxidation Protein Products, Malondialdehyde, Methylglyoxal and Hexanoyl-Lysine. In addition, blood urea, creatinine, triglycerides, potassium, phosphorous, total proteins, glucose, urine protein to creatinine ratio and arterial systemic pressure were also evaluated. After checking for normality, comparisons between groups were performed followed by multiple-comparison tests. P values ≤0.05 were considered significant. Correlations between plasma

concentrations of the considered biomarkers and of the other metabolic parameters were investigated using Spearman's correlation coefficient.

Results (P)

Advanced oxidation protein products, malondialdehyde and hexanoyl-lysine concentrations were significantly higher in CKD and ACE treated groups compared with the control group ($P < 0.05$). The ACE group showed an increase in the levels of carboxymethyllysine when compared with the control group, whereas intermediate values of these biomarkers were found in the CKD group ($P < 0.05$). The highest values of carboxymethyllysine, advanced oxidation protein products and hexanoyl-lysine were found in the ACE treated group. By contrast, the CKD group showed the highest concentration of malondialdehyde. No statistically significant difference was found in the levels of pentosidine or methylglyoxal. Carbonyl stress and end-product formation correlated with creatinine and urea and with each other. Neither pentosidine nor methylglyoxal showed any correlation with other uremic toxins.

Conclusions and relevance

Significantly high concentrations of both intermediates and "end products" of carbonyl/oxidative stress, which are also uremic toxins, were detected in CKD cats. To date, the present study is the first to have concurrently taken into account several uremic toxins and biochemical parameters in cats affected by chronic kidney disease.

Introduction

The term "chronic kidney disease" (CKD) indicates an irreversible decrease in renal function, due to structural or functional defects in one or both kidneys.[1,2] Cats, especially in geriatric age, are commonly affected by CKD.[3,4] In human medicine, it has been shown that oxidative stress (OS) is associated with uremia.[5] It has been hypothesized that two main events occur in CKD: the facilitation of inflammation and oxidative stress[6] by uremic toxins: and the impairment of antioxidant enzymes in uremia.[7,8] To our knowledge, only four studies evaluated oxidative stress in cats diagnosed with CKD: Yu and Paetau-Robinson[9] conducted research on oxidative stress and the effects of dietary antioxidant supplementation; Keegan and Webb [10] correlated oxidative stress parameters with neutrophil function; Krofic Zel et al[11] evaluated the activity of antioxidant systems; and Whitehouse et al[12] investigated the increase in urinary F2-Isoprostanes in different International Renal Interest Society (IRIS) stages of CKD.

OS is a complex phenomenon, which includes many pathways. Although it can be measured in different ways, the assessment of the by-products of OS reactions by means of biomolecules, like proteins, lipids and sugars, is the most common procedure. When reactive species act on lipids many peroxidation products are created, such as hydroperoxides, conjugated dienes, isoprostanes and derived carbonyls, such as malondialdehyde (MDA) and hydroxynonenal

(HNE).[13] It has recently been shown that hexanoyl-lysine (HEL), another marker of fatty acid oxidation, is formed at an earlier stage of the cascade. This compound is a specific marker of omega-6 oxidation and promotes the formation of adducts from linoleic acid or arachidonic acid.[14] Proteins are other molecules sensitive to the action of reactive species. Amino acids (AA) are prone to many reactions, including hydroxylation, nitration, sulphoxidation, chlorination, cross-linking and conversion to carbonyl derivatives.[13]

Of all the by-products originating from proteins damaged by OS, carbonyls, advanced oxidation protein products (AOPP) and adducts formed between AA and free carbonyls or lipid oxidation/reducing sugar products are the most extensively studied.[13] The latter group of reactions consists of the addition of compounds deriving from glycation and lipid peroxidation to proteins:[15] these reactions are also known as carbonylation. This modification is sustained by a surplus of reactive carbonyl compounds; in this circumstance, a series of different complex reactions lead to the formation of more stable final products, called "advanced end-products", such as pentosidine (Pent) and carboxymethyllysine (CML). While Pent is a marker of the glycoxidative cascade and can be classified as an advanced glycation end product (AGE), CML is not only formed along this pathway but also during the lipoxidation cascade; thus, CML can also be considered an advanced lipoxidation end product (ALE) compound.[16] An increase in these reactions is referred to as carbonyl stress and is related to the pathogenesis of several diseases, including chronic renal failure.[17]

From this brief summary, it is clear that OS creates a series of reactions leading to measurable by- and end-products; what it is known in human medicine is that reactive oxygen species (ROS) increase carbonyl stress[18] and facilitate the formation of end-products, which themselves act as inducers of ROS in a vicious cycle (Figure 1). This tight relationship is present in uremic human patients affected by CKD, who show an increase in both carbonyl and oxygen reactive species.[5,7] These molecules are recognized as uremic toxins and may play a role in the progression of chronic renal failure.

Over recent years, research into uremic toxicity has highlighted dozens of retention solutes that interact negatively with physiological mechanisms.[19] Looking at the uremic database,[20] the only uremic toxins studied in feline medicine are creatinine (used for IRIS staging[???]), urea and MDA.[9] Understanding how these substances are formed in chronic diseases can lead to the development of new therapeutic strategies. Currently, in cats affected by CKD, the main therapeutic goal attempts to reduce the progression of the disease.[21] To date, the available approaches are based on the administration of proper diets (with the essential nutritional characteristics established by the Commission Regulation (EU) No 1123/2014) consisting of high quality proteins in reduced quantity and restricted levels of phosphorous). Moreover, according to other sources, this dietary regime should be supplemented with n-3 PUFA and antioxidants, dehydration should be corrected and drugs (such as calcium channel blockers and inhibitors of the renin-angiotensin-aldosterone system, i.e. angiotensin converting enzyme inhibitors-ACE or angiotensin receptor blockers

ARB)[22] introduced to improve renal function, reduce blood pressure, and lower systemic oxidative stress.[21] With regard to this last clinical recommendation, we previously reported that oral antihypertensive therapy exerts antioxidant activity, which scavenges reactive oxygen species in humans.[23]

The aim of the present study is to confirm whether in cats affected by CKD, carbonyl stress and end product formation are higher than in healthy cats and to assess whether ACE inhibitors may affect these hallmarks.

Materials and Methods

Selection of cases

The study was carried out between January 2013 and June 2014. Twenty-two adults cats (9 neutered males and 13 neutered females) aged 4 to 14 years were enrolled. For each cat, a complete anamnesis was obtained and a physical examination, complete blood count (CBC), serum biochemistry and urinalysis performed. Residual samples from routine visits not related to the study were employed. Cat owners gave their consent to the use of surplus samples after routine testing.

The cats were divided into three groups: controls: CKD: and ACE. Animals comprising the control group (CG, n = 6) were enrolled during annual check-up examinations; the inclusion criteria for control animals were based on their clinical history and the absence of any disease on the basis of their anamnesis, physical examination, blood and urine analyses and the absence of medications except for parasitic control. The CKD group consisted of cats with

chronic kidney disease (n = 11) that had not been treated with any drugs in accordance with the clinician's recommendations and had been kept on a renal diet formulated by a diplomat from the European College of Veterinary and Comparative Nutrition (ECVCN). The recruitment of these cats was based on the diagnosis performed by a clinician and on the guidelines for IRIS staging of chronic kidney disease.[22] The third group (ACE, n = 5) included cats affected by CKD, which were being treated with the specific diet and the ACE inhibitor hydrochloride (dosage regime was adapted and based on the values commonly recommended in clinical animal practice), administered for at least 40 days prior to sample collection. The inclusion criteria for CKD cats were based on a stable CKD history (in at least two separate time points) entailing anamnesis, physical examination, measurements of blood creatinine and confirmation of low urine specific gravity (<1035).

The inclusion criteria for all three groups were age (adult, centred on the mean age of the CKD cats) and Body Condition Score (BCS, 9 point scale, according to the American Animal Hospital Association, centred on the mean value for the CKD cats).

The exclusion criteria were the following: pre-renal or post renal azotemia, acute renal injury, acute infections, feline lower urinary tract disease (FLUTD), systemic metabolic disease (e.g., hyperthyroidism), diabetes, heart failure and positivity for feline leukaemia virus (FeLV) or feline immunodeficiency virus (FIV).

Sampling

Serum was collected and stored for 30 minutes at room temperature. Subsequently, it was separated by centrifugation (2500 *g* for 8 minutes) and two aliquots were obtained: one for the analysis of the biochemical and metabolic parameters, mainly related to the renal function, and one for the assessment of the carbonyl stress biomarkers, which constituted the target of the present study. Samples were stored at -80°C and analysed according to the procedures described in the following paragraphs. Urine samples were collected by cystocentesis when required by the clinician or by a non-invasive method (using a urine collection kit) and analysed within one hour by an automated analyser to obtain the urine protein to creatinine (UP/UC) ratio. Blood urea, (UREA), triglycerides (TG), potassium (K), phosphorous (P), creatinine (CREA) and total proteins (TP) were evaluated by an automated analyser. Plasma glucose (Glu) was determined in blood heparinised samples centrifuged within 15 minutes after sample collection.

Systemic arterial pressure (SAP) measurement was taken using an indirect Doppler method via the radial pulse with the cat sitting or in sternal recumbency. The recorded value is the mean of five measurements.

Advanced end-products

All samples were analysed in duplicate.

Pentosidine (PENT)

Detection of pentosidine (PENT) was performed using high performance liquid chromatography (HPLC), according to Valle et al,[24] using a Waters system (Waters S.P.A., Milan, Italy). Briefly, protein content, after delipidation with hexane and precipitation with trichloroacetic acid, was hydrolysed with 6 mol/L hydrochloric acid for 18 h at 110°C in borosilicate screw-capped tubes, dried in a Speed-Vac concentrator and then reconstituted in HPLC-grade water containing 0.01 mol/L heptafluorobutyric acid (HFBA). Subsequently, it was filtered through a 0.45-µm pore diameter Ultrafree MC (Millipore, Milan, Italy) and injected into a Xterra C18 MS column (250 × 4.6 mm; Waters S.P.A., Milan, Italy) with a curvilinear gradient program of 20% – 40% methanol from 0 to 30 min and containing water (MilliQ, Millipore, Milan, Italy); both water and methanol contained 0.01 mol/L HFBA as a counterion. The PENT peaks were monitored using a Waters 2475 fluorescent detector (excitation 335 nm and emission 385 nm). A PENT synthetic standard (prepared as described by Grandhee and Monnier[25]) was injected at the start of each run to determine PENT concentration in the sample using peak area comparison. The amount of PENT was expressed as pmol per mg of plasma protein content.

Carboxymethyllysine (CML)

Serum Carboxymethyllysine (CML) was evaluated by ELISA (EIAab, Wuhan, China), according to the manufacturer's instructions as reported by Bruynsteen et al.[26] The detection range of the CML ELISA kit was 0.78-50 ng/ml, therefore the serum samples were diluted 1:10. Absorbance was read at 450 nm using a microplate reader. The observed results were expressed as ng/ml.

Advanced oxidation protein products (AOPP)

Determination of AOPP was based on spectrophotometric analysis according to Bruynsteen et al.[26] AOPP concentration was measured by spectrophotometry on a microplate reader at λ 340 nm and was calibrated with a chloramine-T (CT) solution in presence of potassium iodide; briefly, 200 µl of serum (diluted 1:10 with PBS) were placed on a 96-well microtiter plate, and 20 µl of acetic acid were added. In standard wells, 10 µl of 1.16 mol/L potassium iodide were added to 200 µl of CT solution (0–100 µmol/L) followed by 20 µl of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 200 µl of PBS, 10 µl of potassium iodide, and 20 µl of acetic acid. The AOPP concentrations were expressed as µmol/L of CT equivalents.

Carbonyls from the peroxidation cascade

Malondialdehyde MDA

Serum malondialdehyde was measured by HPLC according to the method published by Nielsen et al,[27] with slight modifications. Briefly, aliquots of serum were mixed (volume/volume) with a 0.6% (w/v) aqueous solution of thiobarbituric acid (TBA). The mixture was acidified with 1/20 volume of 100% (w/v) trichloroacetic acid and heated at 100°C for 1 hour. The samples were then cooled in ice and centrifuged at 13.000 *g* for 5 minutes. Aliquots of 50 ul of the supernatant were injected into the HPLC system equipped with a Novapak C18 4µm 3.9x150 mm column (Waters S.P.A, Milan, Italy). The elution was

252 isocratic. The mobile phase consisted of a mixture of a 10 mM potassium
253 dihydrogen phosphate solution, adjusted to pH 6.8 with KOH 1M, and
254 methanol in a ratio of 60/40. The flow rate was 1 ml/min. Detection was
255 performed by a spectrofluorometery (Ex/Em = 532/553 nm). Under our
256 conditions, the peak of the MDA-TBA adduct was well resolved at a retention
257 time of 4.8 min.
258 MDA concentration (nmol/ml) was calculated in reference to a calibration curve
259 of MDA sodium salt standard according to the methodology developed by Nair
260 et al[28]. Concentration was expressed in nmol/ml.
261
262 *Hexanoyl-Lysine HEL*
263 Hexanoyl-Lys (HEL) was evaluated by ELISA (JalCA., Shizuoka, Japan),
264 according to the manufacturer's instructions. The detection range of the HEL
265 ELISA kit was 2-700 nmol/L. After overnight incubation with alpha-
266 chymotrypsin, serum samples were ultrafiltered (cut-off 10kDa) and diluted 1:2.
267 Absorbance was read at 450 nm. Results were expressed as nmol/mg protein.
268
269 ***Carbonyl from the glycoxidation cascade***
270 *Methylglyoxal MGO*
271 Methylglyoxal was evaluated according to the method proposed by Wild et al,[29]
272 with slight modifications. The method is based on the reaction between N-
273 acetyl-L-cysteine (Sigma Aldrich) and methylglyoxal at room temperature. The
274 reaction was performed in 100 mM sodium dihydrogen phosphate buffer

(adjusted to pH 7.0 with NaOH 10 M) at 22 °C. As the standard curve for the reaction, different concentrations of MG [???] (0.5, 1, 2, and 5 mM) were used. MG solutions (Sigma Aldrich) equating to 0.5, 2 and 5 mM were added to a volume of 980 μL with sodium dihydrogen phosphate. The reaction was started by adding 20 μL of 500 mM N-acetyl-L-cysteine and the absorption was recorded after 7 minutes. The condensation product, N-*α*-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl) cysteine was determined by recording the absorption at 288 nm (UVIKON 923, Bio-Tek Instrument). Results were expressed as μmol/ml.

Serum protein content determination

Serum protein content was determined using the BCA protein assay kit according to the manufacturer's instructions (Thermo Fisher Scientific., Rockford, IL, USA).

Statistical analysis

Data were analysed using GraphPad Prism for Mac ,version 7.00 (GraphPad Software, La Jolla California USA, www.graphpad.com).

All measurements were performed in duplicate and data were expressed as medians and interquartile ranges. After checking for normality using the Kolmogorov-Smirnov test, comparisons between groups were performed using the Kruskal–Wallis test followed by Dunn's multiple-comparison tests. P values <0.05 were considered significant (a "tendency" was considered for $P < 0.1$).

Relationships between plasma concentrations of the considered biomarkers and the other metabolic parameters were investigated using the one-tailed Spearman's correlation coefficient (rS).

Results

Results are illustrated in Tables 1 and 2. Comparison of the groups showed a significant increase in Crea and Urea in CKD and ACE treated groups, compared with healthy (CG) cats (Table1). No statistically significant differences were found between groups for TP, GLU, TG and K. The highest concentration of P was found in the CKD group , whereas the ACE treated group showed intermediate values. In both CKD and ACE treated groups UP/UC was higher in comparison with the control group.

Table1. Summary of laboratory findings for selected clinical parameters in CG, CKD and ACE treated cats, respectively. Data are reported as medians plus interquartile range (25th and 75th percentiles). Letters identify differences between group comparisons (P < 0.05).

	CG	CKD	ACE
CREA	1.20[a] (1.05;1.48)	3.70[b] (1.89;6.0)	2.10[b] (1.95;3.40)
UREA	42.0[a] (34.5;54.0)	73.0[b] (59.0;188.0)	125.0[b] (91.0;166.5)
TP	6.20 (5.95;7.05)	6.30 (5.7;7.0)	6.80 (5.55;7.10)
GLU	101.0 (99.0;103.5)	96.0 (83;107.0)	90.0 (79.0;120.0)
TG	29.0 (23.0;49.0)	44.0 (37.0;45.0)	35.0 (28.0;48.50)
K	4.30 (3.55;4.55)	4.90 (3.80;5.55)	4.50 (4.30;6.35)
P	4.20[a] (4.0;5.65)	7.3[b] (5.15;8.0)	5.10[ab] (5.0;5.50)
UP/UC	0.21[a] (0.20;0.27)	0.70[b] (0.40;0.74)	1.60[b] (1.10;3.10)
SAP	145.0 (140.0;155.0)	150.0 (140.0;230.0)	155.0 (135.0;187.50)

CREA: creatinine (mg/dl); TP: total proteins (g/dl); GLU: glucose (mg/dl); TG: triglycerides (mg/dl); K: potassium (mEq/l); P: phosphorus (mEq/l); UP/UC: urine protein to creatinine ratio; SAP: systemic arterial pressure (mmHg).

AOPP, MDA and HEL concentrations were significantly higher in CKD and ACE treated groups in comparison with the control (CG) group. When compared with CG, CML was higher in ACE, whereas CKD showed intermediate values. ACE treated groups were characterized by the highest values of CML, AOPP and HEL; conversely, the CKD group showed the highest concentration of MDA. The levels of PENT and MGO showed no statistical differences between groups (Tab.2).

Table 2 Advanced glycated end-products and carbonyl compounds in CG, CKD and ACE cats. Data are reported as medians plus interquartile range (25th and 75th percentiles). Letters identify differences between group comparisons (P < 0.05)

	CG	CKD	ACE
CML	13.81[a] (11.64;19.46)	25.34 [-](21.89;43.08)	42.85[b] (33.11;65.43)
AOPP	83.61[a] (66.2;103.9)	189.3[b] (120.2;288.6)	247.1[b] (137.0;368.9)
PENT	2.23 (0.63;5.77)	1.47 (1.09;4.33)	1.47 (1.28;3.89)
MGO	360.90 (226.20;531.00)	283.40 (177.61;362.00)	261.00 (240.30; 370.70)
MDA	4.85 [a] (3.80;8.79)	27.02[b] (17.74;48.85)	24.70[b] (15.42;58.21)
HEL	0.26[a] (0.16;0.35)	0.88[b] (0.62;1.12)	1.26[b] (0.43;2.31)

CML: carboxymethyllysine (ng/ml); AOPP: advanced oxidation protein products (µmol/L of CT equivalents/mg protein); PENT: pentosidine (pmol/mg protein); MGO: methylgyoxal (µmol/ml); MDA: malondialdehyde (nmol/ml); HEL: hexanoyl-lysine (nmol/mg protein).

Crea was positively correlated with CML (r_s 0.49, $P < 0.05$), AOPP (r_s 0.56, $P < 0.05$), MDA (r_s 0.47, $p<0.05$), and HEL (r_s 0.50, $p<0.05$). It was also correlated with laboratory findings for selected clinical parameters as Urea (r_s 0.79, $P < 0.0001$), P (r_s 0.57, $P < 0.05$) and UP/UC (r_s 0.64, $P < 0.01$). Urea was positively correlated to CML (r_s 0.46, $P < 0.05$), AOPP (r_s 0.62, $P < 0.01$), MDA (r_s 0.72, $P < 0.0001$), and HEL (r_s 0.56, $P < 0.05$), as well as to K (r_s 0.52, $P < 0.05$) and UP/UC (r_s 0.65, $P < 0.01$).

HEL was positively correlated with CML (r_s 0.48, $P < 0.05$), AOPP (r_s 0.76, $P < 0.0001$), MDA (r_s 0.90, $P < 0.0001$) and K (r_s 0.60, $P < 0.01$).

AOPP was positively correlated with CML (r_s 0.56, $P < 0.05$), MDA (r_s 0.75, $P < 0.0001$), P (r_s 0.48, $P < 0.05$) and UP/UC (r_s 0.54, $P < 0.05$).

MDA was positively correlated with K (r_s 0.65, $P < 0.01$) and UP/UC (r_s 0.54, $P < 0.01$) and to CML (r_s 0.36, $P = 0.05$). CML was negatively correlated with GLU (r_s -0.482, $P <0.05$). MGO was negatively correlated with K (r_s -0.574, $P < 0.05$). PENT was only positively correlated with SAP (r_s 0.46, $P < 0.05$).

The correlation table is provided as supplementary data.

Discussion

The present study focuses on hallmarks of oxidative stress and carbonyl stress in feline CKD. Significantly higher concentrations of intermediates and "end products" of carbonyl/oxidative stress, which are also uremic toxins, were detected in nephropathic cats.

An increased concentration of creatinine and urea and an increase in the urine protein to creatinine ratio (UP/UC, indicating proteinuria associated with kidney disease, provided that pre-renal and post-renal causes are excluded) were observed in CKD and ACE treated cats. ACE inhibitors, like benazepril, have been shown to reduce proteinuria in cats[30]; however, in the present study, UP/UC in the ACE treated group was even higher than in the other group. The late initiation of the therapy (introduced 40-60 days before sample collection) can probably explain such a difference.

Over 100 substances have been classified as uremic toxins by the European Uremic Toxin (EUTox) Work Group[20] and recent studies have thoroughly categorised these different molecules. Urea and creatinine are the most common uremic toxins that can increase in cats with CKD and, as expected, in our study both of them increased in the cats with CKD. These compounds are soluble in water, have low molecular weights, and are classified by EUTox as the most reliable biomarkers for the evaluation of renal failure.[31] Not surprisingly, in our study, Urea and Crea showed a positive correlation with each other as well as with UP/UC. In fact, an increase in these parameters typically occurs in CKD cats, and it is used as a diagnostic tool according to the IRIS staging of CKD[22].

However, several other metabolites, other than these compounds, contribute to the toxic environment caused by the disease.[31] Their concentrations provide insight into the clinical severity of CKD and favour the maintenance of both oxidative and carbonyl stress in a vicious circle.

In addition, Crea and Urea positively correlated with CML, AOPP, MDA and HEL, confirming an association with two recognized markers of renal failure in feline medicine. In particular, a statistically significant increase of CML, AOPP, HEL and MDA was found in the cats affected by CKD. By contrast, they showed a negligible increase in PENT and MGO. These differences were also consistently observed in the ACE treated group.

AOPP are a cluster of oxidative products derived from proteins and are recognised as markers of protein oxidative damage and of inflammation severity. The injured proteins are generated through a mechanism involving free radical direct oxidation of amino acids (e.g., tyrosine (Tyr), lysine (Lys), proline (Pro), arginine (Arg), etc.) (Dean et al 1997 [This should be added to the References]), and as an indirect consequence of lipoperoxidation.

AOPP are also defined as "accumulated solutes, normally excreted by the kidneys, that interact negatively with biological functions".[15]

It has been reported that serum AOPP concentration (closely correlated with other markers) increases with the progression of chronic diseases.[32,33] Accumulation of plasma and renal AOPPs is a common pathologic finding in human patients with CKD.[34] Witko-Sarsat et al[32,33] showed that *in vivo* levels of AOPP correlated well with creatinine clearance.

In human studies of uremic patients, the concentration of plasma AOPP is related to the oxidative activity of circulating neutrophils, suggesting that these leukocytes might be involved in plasma AOPP formation through the myeloperoxidase/H_2O_2 system. In agreement with this hypothesis, Keegan and

403 Webb[10] reported that the neutrophil oxidative burst is higher in chronic renal
404 failure. Moreover, recent studies showed alterations in neutrophil oxidative
405 metabolism and oxidative stress in dogs with CKD.[35–39]
406 Neutrophils can, therefore, be a source of pro-oxidant molecules contributing to
407 an abnormal production of ROS and participating to the formation of AOPPs.
408 Neutrophil oxidative metabolism can, in turn, be activated by other uremic
409 toxins.[39]
410 In accordance with the above mentioned findings, our study demonstrates for
411 the first time a significant increase in AOPP in cats. In fact, when compared with
412 control animals, CKD and ACE treated cats showed a 77% and 132% increase,
413 respectively, in this parameter. AOPP are good hallmarks of the progression of
414 chronic renal failure and the severity of uremia;[40] accordingly, in our study their
415 concentrations correlated well with those of creatinine and urea. As previously
416 reported, they are also a good and accurate biomarker of oxidative stress,[41] and
417 in the present study significant correlations with other markers of
418 lipoperoxidation were found (CML, MDA, HEL).
419 Serum MDA [???] is an organic and very simple compound and one of the highly
420 reactive carbonyls originating from PUFA oxidation (in particular from
421 peroxidation of arachidonic, eicosapentaenoic and docosahexaenoic acid).[42] In
422 humans, it is the most abundant product, since it comprises 70% of all the
423 carbonyls obtained by lipid peroxidation.[18]
424 In this study, serum MDA consistently increased in CKD (284%) and in ACE
425 treated (256%) cats. An increase in serum MDA was previously observed in CKD

426 cats by Yu and Paetau-Robinson[9] and four weeks of antioxidant supplements did
427 not exert any effect on this parameter.
428 MDA correlates with AOPP, HEL and with other markers of CKD (creatinine,
429 urea, Potassium and PU/CU): the remarkably high correlation with HEL (r =
430 +0.904) is explained by their shared origin from lipid peroxidation.
431 Since potassium did not vary significantly between groups but it often varies in
432 CKD cats[43] according to diet intake, the positive correlation with MDA is
433 probably suggestive of an increased oxidative stress in cats with higher
434 potassium quartiles, although still in the normal range.
435 Hexanoyl-lysine (HEL) is a recently discovered lipid peroxidation biomarker
436 derived from the oxidation of omega-6 unsaturated fatty acids.[14] HEL is formed
437 when a lipid hydroperoxide links to a lysine residue, forming a stable
438 compound.[44] Arachidonic acid is one of the PUFA that, after oxidation, gives rise
439 to MDA and HEL compounds;[42] it is often added to cat food, especially during
440 growth, gestation and lactation, because these animals are unable to synthesize
441 it.[45]
442 The actual estimated requirements, based on a low reported synthesis capacity,
443 is 8 mg/100 g dry matter [???] (DM), considering a metabolic energy requirement
444 (MER) of 75 kcal/kg^{0,67}[???] in adult animals and 20 mg/100 g DM during growth
445 and reproduction[46]. Arachidonic acid is naturally present in animal tissues, so it
446 does not have to be added to food containing proteins of animal sources.[45]
447 When compared with control cats, CKD and ACE treated cats showed highly
448 significant increases in serum HEL (three times higher than controls in the CKD

group and five times higher than controls in ACE cats). Therefore, it would be interesting to evaluate whether a high content of arachidonic acid in the diet of CKD cats might generate oxidants and promote oxidative stress, as observed in CKD disease; if that were the case, more attention should be paid to the total amount of this substance provided to cats, in particular to those affected by CKD. The diet should be balanced with an equivalent amount of antioxidants to avoid the increase of carbonyls, such as MDA and HEL, originating from the peroxidation cascade.

CML is formed during the Maillard reaction by a process of glycoxidation. It can derive from different compounds, such as aldoses, ketoses, ascorbate, PUFAs and other molecules, and it is classified as an advanced glycation-lipoxidation end-product (AGE). Another relevant source of CML is from food. It is found in dairy products, but also in meat, fish, cereal-derived products, and in a group of fruit and vegetables that have been cooked or treated in an industrial context.[16]

As previously shown[47], CML increases in uremic patients and such an increase is also generally paralleled by increased levels of PENT,[47] since they share the same molecular origin. In the present study, CML levels increased 3-fold in the ACE treated group and doubled in the CKD group, but, intriguingly, a correlation with PENT was not observed.

We might hypothesize that although CML mainly derives from the peroxidation cascade, diet may also play a noteworthy role. Hull et al[48] showed that the CML content of cat food can be high. High exposure to CML should be

472 taken into careful consideration because this compound could be hazardous for
473 feline health. This is even more true in cats with CKD, since CML seems to be
474 associated with degenerative disorders and chronic kidney diseases.[49]
475 Interestingly, CML levels are positively correlated with markers of kidney
476 function, such as Crea and Urea, and negatively correlated with serum glucose,
477 indicating that, in cats, high glucose might not lead to the formation of AGEs
478 through the Maillard reaction. Other significant correlations were found with
479 HEL ($P < 0.05$) and AOPP ($P < 0.05$), which can therefore be included, as is the
480 case for humans, in the array of toxins found in uremic cats.
481 Methylglyoxal is generated by a series of metabolic pathways, mostly belonging
482 to the glycolytic process. It is an important precursor of advanced glycation end
483 products, being a highly powerful glycating agent. It is also involved in diabetic
484 microvascular complications.[50] Increases in MGO have been observed during
485 hyperglycaemia as well as in the uremic state.
486 In our study, MGO was not significantly different between the three groups of
487 cats. Therefore, we can hypothesize that, in contrast to what has been observed
488 in humans [perhaps a reference should be added here], the glycation pathway
489 does not play a role in the pathophysiology of uremia in cats.
490 Pentosidine is a well-known advanced glycation end product and a uremic toxin,
491 that, surprisingly, the levels of which were not, surprisingly, significantly
492 different in the three groups of our study. This finding differs from what has been
493 reported in human patients affected by CKD. In these cases a marked increase of
494 pentosidine was found and also associated with a low glomerular filtration rate,

oxidative stress and inflammation.[51–53] In our opinion, in feline CKD, lipids and lipoperoxidation seem to play a more important role than glucose, glycation or glycoxidation, which seem to be unrelated to this disease. In cats, a distinct pathway for the formation and accumulation of uremic toxins should be considered, along with different uremic oxidative stress compounds. In line with this reasoning, PENT might turn out to be a minor end product in the bulk of AGEs.

In addition, in our groups of cats, PENT does not correlate with other carbonyl determinations and other clinical parameters, although it does with SAP. We can hypothesize that, in cats, pentosidine accumulation is, as in humans: age related: connected with the progression of renal failure: and occurring mainly in tissues rather than in blood. Tissue accumulation of PENT is well described in humans and in other animals such as rats with CKD (where it accumulates in the tubules),[54] dogs, rabbits, monkeys, etc. Moreover, in cats with CKD, an interstitial fibrosis has been observed.[55] Pentosidine accumulation may occur in the kidneys or in the artery walls, contributing to an increase in blood pressure. It is noteworthy that, in humans, serum pentosidine is positively associated with arterial stiffness and thickness.[56] Further studies are needed to evaluate this intriguing hypothesis.

According to our results, the use of ACE inhibitors exerted a negligible effect on the carbonyl oxidative stress status. By contrast, Monacelli et al[23] reported, in humans, that valsartan, an angiotensin II receptor antagonist, besides having antihypertensive activity, is also effective in scavenging oxidative stress species.

However, some differences between the present study and that of Monacelli et al[23] should be considered. Our experimental animals only received the therapy for 40-60 days, while the trial reported by Monacelli et al[23] lasted 6 months. The difference in the duration of the treatment may explain the lack of efficacy reported in our study. Such a difference could also be due to the use of a different type of drug. In fact, in our experiment cats were treated with benazepril, an angiotensin-converting-enzyme inhibitor, whereas in the study carried out by Monacelli et al[23], human patients received valsartan, an AT1 antagonist.

Although carefully designed, our study suffers from some limitations, mainly due to its small sample size. In particular, it would have been more appropriate to enrol a larger number of cats for each IRIS stage (from 1 to 4) in order to draw more valid conclusions. The progression of uremic toxin production and/or the existence of a CKD threshold for their formation is also a matter of debate and deserves further investigation. Even if we had just included cats fed a renal diet, we would still have had confounding effects. In fact, renal diets of different brands can also vary in terms of omega 3-6, protein, phosphorus and carbonyl content. Further studies focusing on the intake of single diet components are required.

Conclusions

To the best of our knowledge, this is the first study to take into account contemporaneously several uremic toxins - according to the EUTox database - and biochemical parameters in cats affected by CKD. Evidence of strong carbonyl

stress is confirmed in CKD cats, irrespective of the therapy with ACE inhibitors. These toxic molecules contribute to maintaining and promoting oxidative stress and facilitate the progression of systemic damage. However, two markers Pentosidine and Methylglioxal remained unaffected. This phenomenon suggests some hypotheses that need to be verified and, at the same time, raises the possibility that the disease might be characterized by a new pattern of markers. The significant and striking increases in CML and HEL offer challenging possibilities in terms of specific diets aimed at the prevention of kidney disease. Currently, more studies are needed to clarify the disease mechanisms and their associations with clinical signs, cellular damages and kidney malfunction in affected cats.

The results of the present study broaden our understanding of this widespread problem afflicting feline health and help pave the way towards new research fields required to make substantial progress in clinical veterinary practice.

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Authors' note

Part of the data were presented at the 2015 ESVCN conference.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship and publication of this article.

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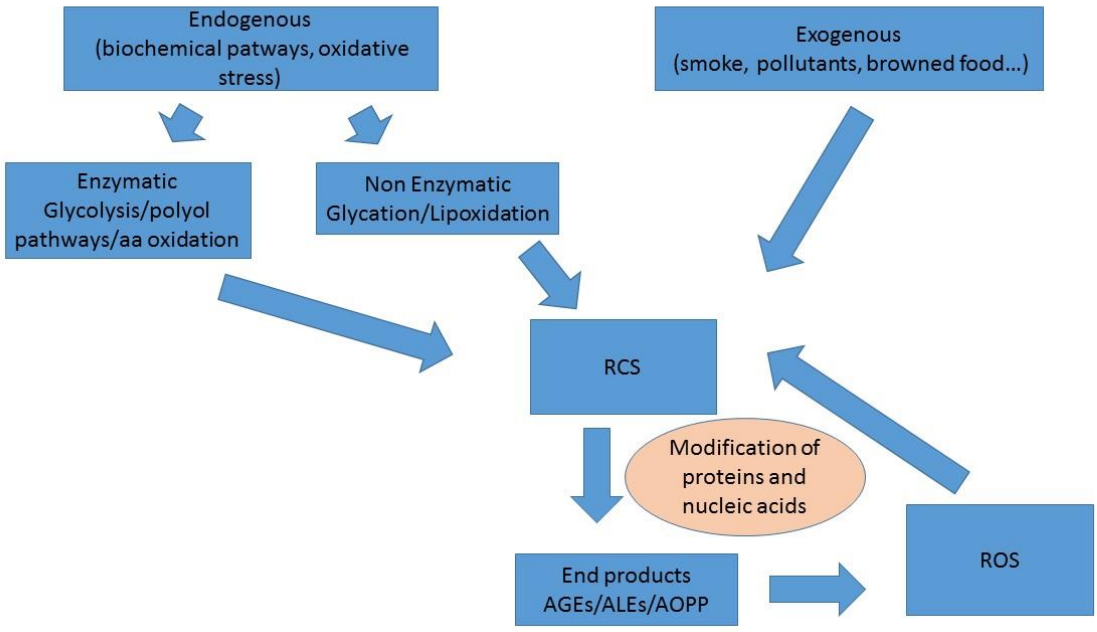
740 **Figure 1. NB note misspelling of "pathways" in top left box.**



741

742 ***Figure 1. The vicious cycle of Reactive Carbonyl Species (RCS) and Reactive Oxygen Species (ROS)***
743 ***formation pathways****. AGEs: advanced glycation end products; ALEs: advanced lipoxidation end products; AOPP:*
744 *advanced oxidation protein products; aa: amino acids*

745