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Investigation of hallmarks of carbonyl stress and formation of end products in feline chronic kidney disease as markers of uraemic toxins

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1 **Original Article**

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

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23

24 **Abstract**

25

26 **Objectives**

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

34

35 **Methods**

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

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

49

50 **Results (P)**

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

63

64 **Conclusions and relevance**

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

70

71 **Introduction**

72

73 The term “chronic kidney disease” (CKD) indicates an irreversible decrease in
74 renal function, due to structural or functional defects in one or both kidneys.^{1,2}

75 Cats, especially in geriatric age, are commonly affected by CKD.^{3,4} In human
76 medicine, it has been shown that oxidative stress (OS) is associated with uremia.⁵

77 It has been hypothesized that two main events occur in CKD: the facilitation of
78 inflammation and oxidative stress⁶ by uremic toxins; and the impairment of
79 antioxidant enzymes in uremia.^{7,8} To our knowledge, only four studies evaluated

80 oxidative stress in cats diagnosed with CKD: Yu and Paetau-Robinson⁹
81 conducted research on oxidative stress and the effects of dietary antioxidant
82 supplementation; Keegan and Webb¹⁰ correlated oxidative stress parameters
83 with neutrophil function; Krofic Zel et al¹¹ evaluated the activity of antioxidant
84 systems; and Whitehouse et al¹² investigated the increase in urinary F2-
85 Isoprostanes in different International Renal Interest Society (IRIS) stages of
86 CKD.

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

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

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

115 From this brief summary, it is clear that OS creates a series of reactions
116 leading to measurable by- and end-products; what it is known in human
117 medicine is that reactive oxygen species (ROS) increase carbonyl stress¹⁸ and
118 facilitate the formation of end-products, which themselves act as inducers of ROS
119 in a vicious cycle (Figure 1). This tight relationship is present in uremic human
120 patients affected by CKD, who show an increase in both carbonyl and oxygen
121 reactive species.^{5,7} These molecules are recognized as uremic toxins and may play
122 a role in the progression of chronic renal failure.

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

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

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

145

146 **Materials and Methods**

147 *Selection of cases*

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

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

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

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

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

183

184 *Sampling*

185 Serum was collected and stored for 30 minutes at room temperature.
186 Subsequently, it was separated by centrifugation (2500 g for 8 minutes) and two
187 aliquots were obtained: one for the analysis of the biochemical and metabolic
188 parameters, mainly related to the renal function, and one for the assessment of
189 the carbonyl stress biomarkers, which constituted the target of the present
190 study. Samples were stored at -80°C and analysed according to the procedures
191 described in the following paragraphs. Urine samples were collected by
192 cystocentesis when required by the clinician or by a non-invasive method
193 (using a urine collection kit) and analysed within one hour by an automated
194 analyser to obtain the urine protein to creatinine (UP/UC) ratio. Blood urea,
195 (UREA), triglycerides (TG), potassium (K), phosphorous (P), creatinine (CREA)
196 and total proteins (TP) were evaluated by an automated analyser. Plasma
197 glucose (Glu) was determined in blood heparinised samples centrifuged within
198 15 minutes after sample collection.
199 Systemic arterial pressure (SAP) measurement was taken using an indirect
200 Doppler method via the radial pulse with the cat sitting or in sternal
201 recumbency. The recorded value is the mean of five measurements.

202

203 *Advanced end-products*

204 All samples were analysed in duplicate.

205 *Pentosidine (PENT)*

206 Detection of pentosidine (PENT) was performed using high performance liquid
207 chromatography (HPLC), according to Valle et al,²⁴ using a Waters system
208 (Waters S.P.A., Milan, Italy). Briefly, protein content, after delipidation with
209 hexane and precipitation with trichloroacetic acid, was hydrolysed with 6
210 mol/L hydrochloric acid for 18 h at 110°C in borosilicate screw-capped tubes,
211 dried in a Speed-Vac concentrator and then reconstituted in HPLC-grade water
212 containing 0.01 mol/L heptafluorobutyric acid (HFBA). Subsequently, it was
213 filtered through a 0.45-µm pore diameter Ultrafree MC (Millipore, Milan, Italy)
214 and injected into a Xterra C18 MS column (250 × 4.6 mm; Waters S.P.A., Milan,
215 Italy) with a curvilinear gradient program of 20% – 40% methanol from 0 to 30
216 min and containing water (MilliQ, Millipore, Milan, Italy); both water and
217 methanol contained 0.01 mol/L HFBA as a counterion. The PENT peaks were
218 monitored using a Waters 2475 fluorescent detector (excitation 335 nm and
219 emission 385 nm). A PENT synthetic standard (prepared as described by
220 Grandhee and Monnier²⁵) was injected at the start of each run to determine
221 PENT concentration in the sample using peak area comparison. The amount of
222 PENT was expressed as pmol per mg of plasma protein content.

223 *Carboxymethyllysine (CML)*

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

229 *Advanced oxidation protein products (AOPP)*

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

241

242 *Carbonyls from the peroxidation cascade*

243 *Malondialdehyde MDA*

244 Serum malondialdehyde was measured by HPLC according to the
245 method published by Nielsen et al,²⁷ with slight modifications. Briefly, aliquots
246 of serum were mixed (volume/volume) with a 0.6% (w/v) aqueous solution of
247 thiobarbituric acid (TBA). The mixture was acidified with 1/20 volume of 100%
248 (w/v) trichloroacetic acid and heated at 100°C for 1 hour. The samples were
249 then cooled in ice and centrifuged at 13.000 g for 5 minutes. Aliquots of 50 μ l of
250 the supernatant were injected into the HPLC system equipped with a Novapak
251 C18 4 μ m 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 spectrofluorometry (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 al²⁸. 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

275 (adjusted to pH 7.0 with NaOH 10 M) at 22 °C. As the standard curve for the
276 reaction, different concentrations of MG [???] (0.5, 1, 2, and 5 mM) were used.
277 MG solutions (Sigma Aldrich) equating to 0.5, 2 and 5 mM were added to a
278 volume of 980 µL with sodium dihydrogen phosphate. The reaction was started
279 by adding 20 µL of 500 mM N-acetyl-L-cysteine and the absorption was
280 recorded after 7 minutes. The condensation product, N- α -acetyl-S-(1-hydroxy-
281 2-oxo-prop-1-yl) cysteine was determined by recording the absorption at 288
282 nm (UVIKON 923, Bio-Tek Instrument). Results were expressed as µmol/ml.

283

284 *Serum protein content determination*

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

288

289

290 **Statistical analysis**

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

293 All measurements were performed in duplicate and data were expressed as
294 medians and interquartile ranges. After checking for normality using the
295 Kolmogorov-Smirnov test, comparisons between groups were performed using
296 the Kruskal–Wallis test followed by Dunn's multiple-comparison tests. P values
297 <0.05 were considered significant (a “tendency” was considered for P < 0.1).

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

301

302 **Results**

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

310

311 **Table1. Summary of laboratory findings for selected clinical parameters in CG, CKD and**
 312 **ACE treated cats, respectively. Data are reported as medians plus interquartile range**
 313 **(25th and 75th percentiles). Letters identify differences between group comparisons (P <**
 314 **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)

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

318

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

326

327

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

331

	CG	CKD	ACE
CML	13.81 ^a (11.64;19.46)	25.34 ^c (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)

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

335

336 Crea was positively correlated with CML (r_s 0.49, $P < 0.05$), AOPP (r_s 0.56, $P <$
337 0.05), MDA (r_s 0.47, $p < 0.05$), and HEL (r_s 0.50, $p < 0.05$). It was also correlated with
338 laboratory findings for selected clinical parameters as Urea (r_s 0.79, $P < 0.0001$), P
339 (r_s 0.57, $P < 0.05$) and UP/UC (r_s 0.64, $P < 0.01$). Urea was positively correlated to
340 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
341 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, $P <$
342 0.01).

343 HEL was positively correlated with CML (r_s 0.48, $P < 0.05$), AOPP (r_s 0.76, $P <$
344 0.0001), MDA (r_s 0.90, $P < 0.0001$) and K (r_s 0.60, $P < 0.01$).

345 AOPP was positively correlated with CML (r_s 0.56, $P < 0.05$), MDA (r_s 0.75, $P <$
346 0.0001), P (r_s 0.48, $P < 0.05$) and UP/UC (r_s 0.54, $P < 0.05$).

347 MDA was positively correlated with K (r_s 0.65, $P < 0.01$) and UP/UC (r_s 0.54, $P <$
348 0.01) and to CML (r_s 0.36, $P = 0.05$). CML was negatively correlated with GLU (r_s
349 -0.482 , $P < 0.05$). MGO was negatively correlated with K (r_s -0.574 , $P < 0.05$). PENT
350 was only positively correlated with SAP (r_s 0.46, $P < 0.05$).

351 The correlation table is provided as supplementary data.

352

353 **Discussion**

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

358 An increased concentration of creatinine and urea and an increase in the urine
359 protein to creatinine ratio (UP/UC, indicating proteinuria associated with kidney
360 disease, provided that pre-renal and post-renal causes are excluded) were
361 observed in CKD and ACE treated cats. ACE inhibitors, like benazepril, have
362 been shown to reduce proteinuria in cats³⁰; however, in the present study, UP/UC
363 in the ACE treated group was even higher than in the other group. The late
364 initiation of the therapy (introduced 40-60 days before sample collection) can
365 probably explain such a difference.

366 Over 100 substances have been classified as uremic toxins by the European
367 Uremic Toxin (EUTox) Work Group²⁰ and recent studies have thoroughly
368 categorised these different molecules. Urea and creatinine are the most common
369 uremic toxins that can increase in cats with CKD and, as expected, in our study
370 both of them increased in the cats with CKD. These compounds are soluble in
371 water, have low molecular weights, and are classified by EUTox as the most
372 reliable biomarkers for the evaluation of renal failure.³¹ Not surprisingly, in our
373 study, Urea and Crea showed a positive correlation with each other as well as
374 with UP/UC. In fact, an increase in these parameters typically occurs in CKD cats,
375 and it is used as a diagnostic tool according to the IRIS staging of CKD²².

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

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

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

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

394 It has been reported that serum AOPP concentration (closely correlated with
395 other markers) increases with the progression of chronic diseases.^{32,33}

396 Accumulation of plasma and renal AOPPs is a common pathologic finding in
397 human patients with CKD.³⁴ Witko-Sarsat et al^{32,33} showed that *in vivo* levels of
398 AOPP correlated well with creatinine clearance.

399 In human studies of uremic patients, the concentration of plasma AOPP is related
400 to the oxidative activity of circulating neutrophils, suggesting that these
401 leukocytes might be involved in plasma AOPP formation through the
402 myeloperoxidase/H₂O₂ system. In agreement with this hypothesis, Keegan and

403 Webb¹⁰ 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 docosaheptaenoic 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-Robinson⁹ 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 cats⁴³ 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 reproduction⁴⁶. 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

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

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

464 As previously shown⁴⁷, CML increases in uremic patients and such an
465 increase is also generally paralleled by increased levels of PENT,⁴⁷ since they
466 share the same molecular origin. In the present study, CML levels increased 3-
467 fold in the ACE treated group and doubled in the CKD group, but, intriguingly,
468 a correlation with PENT was not observed.

469 We might hypothesize that although CML mainly derives from the
470 peroxidation cascade, diet may also play a noteworthy role. Hull et al⁴⁸ showed
471 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,

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

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

514 According to our results, the use of ACE inhibitors exerted a negligible effect on
515 the carbonyl oxidative stress status. By contrast, Monacelli et al²³ reported, in
516 humans, that valsartan, an angiotensin II receptor antagonist, besides having
517 antihypertensive activity, is also effective in scavenging oxidative stress species.

518 However, some differences between the present study and that of Monacelli et
519 al²³ should be considered. Our experimental animals only received the therapy
520 for 40-60 days, while the trial reported by Monacelli et al²³ lasted 6 months. The
521 difference in the duration of the treatment may explain the lack of efficacy
522 reported in our study. Such a difference could also be due to the use of a different
523 type of drug. In fact, in our experiment cats were treated with benazepril, an
524 angiotensin-converting-enzyme inhibitor, whereas in the study carried out by
525 Monacelli et al²³, human patients received valsartan, an AT1 antagonist.
526 Although carefully designed, our study suffers from some limitations, mainly
527 due to its small sample size. In particular, it would have been more appropriate
528 to enrol a larger number of cats for each IRIS stage (from 1 to 4) in order to draw
529 more valid conclusions. The progression of uremic toxin production and/or the
530 existence of a CKD threshold for their formation is also a matter of debate and
531 deserves further investigation. Even if we had just included cats fed a renal diet,
532 we would still have had confounding effects. In fact, renal diets of different
533 brands can also vary in terms of omega 3-6, protein, phosphorus and carbonyl
534 content. Further studies focusing on the intake of single diet components are
535 required.

536

537 **Conclusions**

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

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

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

555

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560

561 **Authors' note**

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

563

564 **Conflict of interest**

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

567

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570

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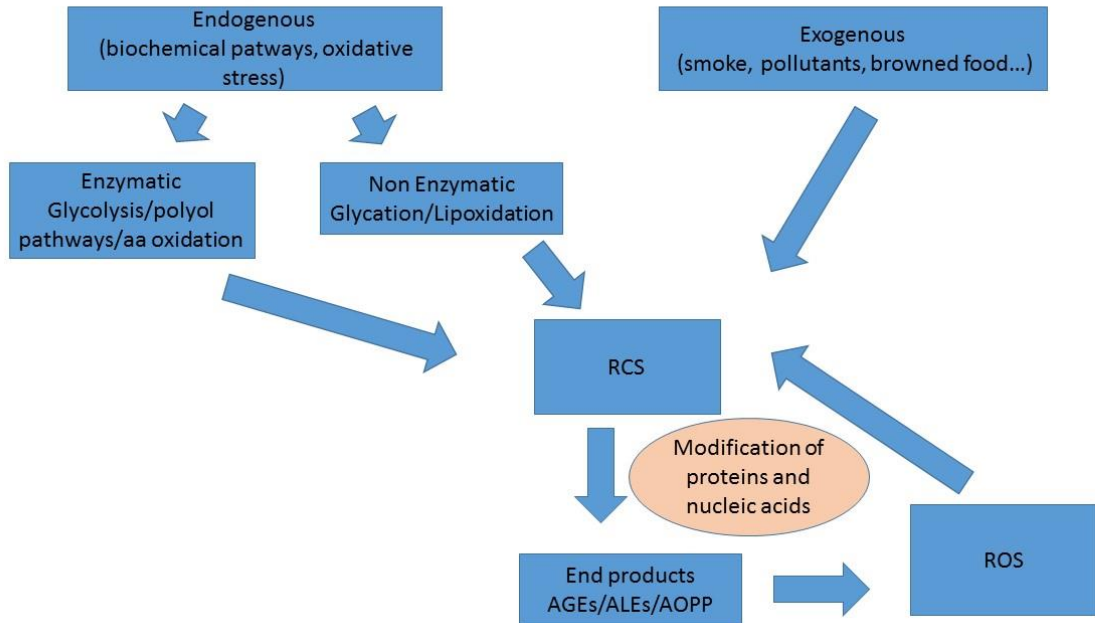
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- 737
- 738

739

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