Manuscript Details

Manuscript number	RVSC_2017_768_R1
Title	Use of urinary gamma-glutamyl transferase (GGT) to monitor the pattern of proteinuria in dogs with leishmaniasis treated with N-methylglucamine antimoniate
Article type	Research Paper

Abstract

The aim of this study was to assess if the coupled analysis of the urinary protein to creatinine (UPC) ratio and of the GGT/UC ratio (the ratio between urinary gamma-glutamyl transferase activity and urinary creatinine) may be used in treated leishmaniotic dogs to differentiate dogs with transient impairment of tubular function from dogs with persistent tubular damage. To this aim, 40 urine from 10 proteinuric and leishmaniotic dogs that at the first visit had high GGT/UC ratio, consistent with tubular damage, were collected and analyzed before treatments and 2, 4 and 6 weeks after treatment with N-methylglucamine antimoniate and allopurinol. Compared with pre-treatment values, at the end of the study period the UPC ratio decreased only in 5/10 dogs, which, however, were still proteinuric or borderline proteinuric. Conversely, the GGT/CU ratio decreased in 8/10 dogs and in 3 of them the values at the end of the study period were below the threshold consistent with tubular proteinuria. The GGT/UC values at 6 weeks was significantly lower than before treatment. However, transient increases were frequent for both the analytes. These results indicate that in most of the dogs that remain proteinuric after treatment, likely due to the persistent glomerular damage, the GGT/UC ratio tends to normalize. This suggests that in these dogs tubular proteinuria at admission depends on functional impairment of tubular cells likely due to the overflow of proteins from damaged glomeruli. However, tubular proteinuria occasionally persists, suggesting that tubulointerstitial damages persist even in dogs responsive to treatments.

Keywords	Canine Leishmaniasis, Proteinuria, Chronic kidney disease, Renal biomarker
Manuscript category	Biochemistry

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There are no linked research data sets for this submission. The following reason is given:
raw data already tabled in the manuscript

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI MEDICINA VETERINARIA

Milano Jan 15th 2017

Dear Editor

Herewith attached you can find the revised version of the manuscript: “Use of urinary ¤-glutamyl transferase (GGT) to monitor the pattern of proteinuria in dogs with leishmaniasis treated with N-methylglucamine antimoniate”, to be considered for publication.

The manuscript has been revised according to the comments of the Reviewer

A revision note, that reports in red the responses to the reviewer comemnts has been uploaded along with the manuscript

Please feel free to contact me for any possible enquiry regarding this manuscript

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RVSC_2017_768

Title: Use of urinary gamma-glutamyl transferase (GGT) to monitor the pattern of proteinuria in dogs with leishmaniasis treated with N-methylglucamine antimoniate

Comments from the editors and reviewers:

-Reviewer 1

We were not able to find comments from reviewer 1. We would be happy to make further changes if comments from Reviewer 1 are provided

-Reviewer 2

Line 24: I suggest to use the term tubular damage than tubular proteinuria

The term “tubular proteinuria” has been replaced where indicated by the reviewer. We did not understand whether the recommendation of the reviewer was to replace the term only at line 24 or throughout the manuscript. For now we have only replaced this term where requested but we would be happy to do it in other parts of the manuscript, if requested.

Line 42: symptoms are descriptive terms used in human medicine where subjective perception occurs

“symptoms” has been replaced by “clinical signs”

Line 98: it is unclear how the authors selected the 10 dogs with Leishmaniasis

This information has been added to the text and the sentences at the beginning of the material and methods section have been modified accordingly

Line 101: 8 males more 3 females = 11 dogs not ten dogs as author described before

We are sorry for this mistake. The error now has been corrected

Line 102: definition of range is not correct. Minimum and maximum is not the range

The paragraph has been modified and the sentence corrected as requested

Line 104: see comment of line 42

“symptoms” has been replaced by “clinical signs”

Line 125-127 It should be better declare numerical aperture of the microscope lens too

This information has been added to the text

Line 131 why authors measure specific gravity by not validated device

The accuracy of the device used in this study was preliminarily assessed in comparison with a validated instrument. This information has now been added to the manuscript

Line 144: there is a “the” more

One of the two “the” has been removed

147: it should be declared better the statistical software (years for example)

Details of the software version have been provided

Line 148: p<0.05 vs P<0.05

We assume that the recommendation of the reviewer is to replace “P” with “*p*”. This change has been done (also in the legend of figure 1).

Line 220: why the authors think that the low number of cases represents a limitation of the study

The low number of cases may reduce the statistical relevance of the results. This explanation has now been added to the manuscript

General comment: Referring to the quotations in lines 43-44-48-51-56-78, it would be more elegant to mention the authors who first highlighted that data

We do not understand how the Reviewer recommends to redistribute the citations within the text. In the current forms citations have been added where appropriate (e.g. where the finding described by each Author) are cited in the text. However, if the Reviewer or the editor prefer a different format for citations we would be happy to modify the citations or to replace them with other citations recommended by the Reviewer

Use of urinary γ-glutamyl transferase (GGT) to monitor the pattern of proteinuria in dogs with leishmaniasis treated with N-methylglucamine antimoniate

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Giulia Mangiagalli, DVM[a]

Fabrizio Ibba, DVM, PhD[a,c]

- Urinary GGT activity may be high in leishmaniotic dogs, due to a tubular injury.
- The high GGT/CU may depend also on a functional impairment of tubular cells
- Leishmanicidal treatments may restore tubular function and decrease the GGT/CU
- In this study the GGT/UC ratio decreased in 8/10 treated dogs
- The GGT/UC ratio may differentiate dogs with permanent or transient tubular injury

Use of urinary γ-glutamyl transferase (GGT) to monitor the pattern of proteinuria in dogs with leishmaniasis treated with N-methylglucamine antimoniate

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Abstract

The aim of this study was to assess if the coupled analysis of the urinary protein to creatinine (UPC) ratio and of the GGT/UC ratio (the ratio between urinary γ-glutamyl transferase activity and urinary creatinine) may be used in treated leishmaniotic dogs to differentiate dogs with transient impairment of tubular function from dogs with persistent tubular damage.

To this aim, 40 urine from 10 proteinuric and leishmaniotic dogs that at the first visit had high GGT/UC ratio, consistent with tubular ~~proteinuria~~damage, were collected and analyzed before treatments and 2, 4 and 6 weeks after treatment with N-methylglucamine antimoniate and allopurinol.

Compared with pre-treatment values, at the end of the study period the UPC ratio decreased only in 5/10 dogs, which, however, were still proteinuric or borderline proteinuric. Conversely, the GGT/CU ratio decreased in 8/10 dogs and in 3 of them the values at the end of the study period were below the threshold consistent with tubular proteinuria. The GGT/UC values at 6 weeks was significantly lower than before treatment. However, transient increases were frequent for both the analytes.

These results indicate that in most of the dogs that remain proteinuric after treatment, likely due to the persistent glomerular damage, the GGT/UC ratio tends to normalize. This suggests that in these dogs tubular proteinuria at admission depends on functional impairment of tubular cells likely due to the overflow of proteins from damaged glomeruli. However, tubular proteinuria occasionally persists, suggesting that tubulointerstitial damages persist even in dogs responsive to treatments.

Keywords: Canine Leishmaniasis; Proteinuria; Chronic kidney disease; Renal biomarker

Introduction

Canine leishmaniasis is characterized by a broad spectrum of ~~symptoms~~clinical signs, due to a variety of lesions in different organs (Paltrinieri et al., 2016). Renal lesions may induce a life-threatening chronic renal disease (CKD) in affected dogs (Koutinas and Koutinas 2014). Leishmaniotic dogs may develop a proteinuric nephropathy that starts with an immune-mediated glomerulonephritis characterized by glomerular proteinuria that in turn induces functional or structural lesions in tubular cells. Therefore, mixed proteinuria is considered the most frequent finding in the advanced stages of canine leishmaniasis (Zatelli et al., 2003). Conversely, leishmanicidal treatments should decrease immuno-complex formation and deposition. This may modify the composition of urinary proteins over time.

The presence and magnitude of proteinuria is considered a risk factor for the progression of renal disease (Jacob et al., 2005) and both the guidelines released by the American College of Veterinary Internal Medicine (Lees et al., 2005) and by the International Renal Interest Society (IRIS Canine GN Study Group, Diagnosis Subgroup, 2013), recommend to monitor proteinuria in dogs at risk for development of glomerular disease and to set up pharmacological treatments as soon as the dog became proteinuric. Moreover, the localization of proteinuria (glomerular vs tubular or both) should be a mandatory step in the investigation of canine proteinuria (Lees et al., 2005). Renal biopsy is the only reliable tool for localizing the renal lesions (Lees et al., 2005). However, the invasiveness of the technique limits its use in the clinical practice, especially when analyses should be repeated over time. Therefore, renal biopsy is recommended only in dogs not responding to treatments or in case of a rapid progression of kidney disease (IRIS Canine GN Study Group Diagnosis Subgroup, 2013). Sodium dodecyl-sulphate polyacrilamide gel electrophoresis (SDS-PAGE) or SDS agarose gel electrophoresis (SDS-AGE) differentiate the origin of proteinuria based on the molecular weight (MW) of urinary proteins (Schultze and Jensen 1998). The SDS techniques correlate with the results of renal biopsy, but their specificity is low (Brown et al., 2010; Zini et al., 2004). Moreover, although

less invasive than renal biopsy, these techniques are not available in routine practices that use in-house analyzers. Recently, the measurement of urinary GGT, with the same analytical principle used on most in-house analyzers, has been proposed as a tool to detect tubular proteinuria in leishmaniotic dogs (Palacio et al., 1997), on which it may predict the results of SDS-AGE (Ibba et al., 2016). The enzyme γ-glutamyl transferase (GGT) is expressed in renal tubular cells (Braun et al., 1983). After detachment of the enzyme from the damaged tubular cells, GGT may be found in urine, where its activity is not influenced by serum levels of GGT since it does not undergo glomerular filtration and GGT activity may be measured using the same analytical principle used in many in-clinic analyzers. Therefore, increases in the ratio between urinary GGT and urinary creatinine (GGT/UC ratio) may early detect renal tubular dysfunction or damage (Brunker et al., 2009; Uechi et al., 1994) pending that GGT activity is measured just after sampling, to avoid storage artifacts (Flandrois et al., 1989).

Based on what reported above, monitoring the magnitude of proteinuria is a milestone of the clinicopathological follow-up in the leishmaniotic patient either at first diagnosis or after treatment (Roura et al., 2013). Changes of protein to creatinine (UPC) ratio in dogs receiving N-methylglucamine antimoniate and allopurinol or allopurinol alone have been already investigated (Pardo-Marin et al., 2017; Pierantozzi et al., 2013; Plevraki et al., 2006). Conversely, to our knowledge, only one study investigated separately the possible changes of urinary markers of glomerular or tubular injury during treatment (Pardo-Marin et al., 2017). However, this latter study was focused on a short post-treatment follow up, and provided contrasting results regarding biomarkers of tubular damage. A more reliable information would be useful in patient's management since the identification of a persistent tubular damage may indicate the progression of canine leishmaniasis while a regression of tubular proteinuria may differentiate dogs with irreversible structural tubular changes from those that before treatment had a functional proteinuria due to the saturation of tubular cells subsequent to the overflow of glomerular proteins.

Hence, the aims of this study were to assess whether the simultaneous analysis of UPC and GGT/UC ratio might provide additional information compared with the traditional approach based on the

evaluation of the UPC ratio alone in leishmaniotic dogs that had tubular proteinuria at admission and successfully responded to treatments with N-methylglucamine antimoniate and allopurinol.

Material and methods

Animals and study design

This study was performed on 40 urine samples from 10 leishmaniotic dogs aged 2 to 11 years (median: 6 years), randomly selected among those referred to our institution for diagnosing, treating and monitoring leishmaniasis and that at the time of first diagnosis were in IRIS stage I (creatinine <1.5 mg/dL). The dogs ~~that~~ were proteinuric (n=9) or borderline proteinuric (n=1) and had a GGT/CU higher than 0.81, i.e. the threshold consistent with a tubular component of proteinuria (Ibba et al., 2016). The study group included 6 crossbreed dogs, 1 Pitbull, 1 German shepherd, 1 Epagneul Breton, 1 English Setter. ~~Eight~~ Seven dogs were males, 3 were females. ~~The age range was 2-11 years (median: 6 years).~~

According to the current guidelines for diagnosis and classification of canine leishmaniasis (Paltrinieri et al., 2010), the diagnosis was based on the presence of typical ~~symptoms~~ clinical signs (e.g. cutaneous lesions, enlarged lymph nodes) and/or laboratory abnormalities (anemia, hyperproteinemia with inverted albumin:globulin ratio and polyclonal gammopathy) and on the detection of amastigotes in cytological specimens from lymph nodes with reactive (pyogranulomatous) lymphoadenopathy. Therefore, serology was not performed in all cases, since, based on the guidelines mentioned above, the detection of intralesional parasites is sufficient to classify the dogs as sick.

Dogs were treated with N-methylglucamine antimoniate (Glucantime, Merial Italia S.p.A., Milan, Italy; 100 mg/kg, SC once a day for 30 days) and allopurinol (Zyloric, Teofarma S.r.l. Pavia, Italy; 10 mg/kg, orally twice a day for 6 months), as recommended by the current guidelines for treatment of canine leishmaniasis (Oliva et al., 2010).

Urine samples were collected, under informed consent by the owners, by cystocenthesis at the time of first diagnosis, before drugs administration, and after 2, 4 and 6 weeks after the beginning of the leishmanicidal treatment. According to the regulations of our Institution, when an informed consent is obtained from the owner, a formal approval from the Ethical Committee is not required if samples are performed for diagnostic or monitoring purposes, as in this case (EC decision 29 Oct 2012, renewed with the protocol n° 02-2016).

Unrinalysis and biochemical tests

Five millilitres of each sample have been centrifuged at 500 g for 5 mins. Then, 4.5 mLs of each supernatant were removed and aliquoted in other tubes.

The remaining 0.5 mLs of each supernatant were used to resuspend the sediment and 50 µLs of the resuspended sediment were examined microscopically at 400× magnification (numerical aperture of the microscope lens = 22), to count the mean number of red blood cells (RBCs) and white blood cells (WBCs) per high power field (hpf) and to exclude that samples had an active sediment (i.e. a sediment characterized by bacteriuria, presence of casts, or with more than 5 RBCs, WBCs, or epithelial cells/hpf).

One aliquot of the supernatant was used to measure GGT activity with the method proposed by Szasz (1969) in an automated spectrophotmeter (Mindray BC-120, Shenzen Mindray Biomedical, Shenzen, China) and to determine the urinary specific gravity (USG) using a manual refractometer (HR-160, Optika SRL, Ponteranica, Bergamo, Italy) whose accuracy was determined in house by comparing the results with those of a refractometer used in a previous study (Rossi et al., 2012). The other

aliquots were frozen (-20°C) to determine the concentration of urinary proteins (UP) and urinary creatinine (UC) within one month from sampling.

Specifically, UPs were measured after thawing with an automated spectrophotometer (Cobas Mira, Roche Diagnostics, Basel, Switzerland) using pyrogallol red (Total Proteins High Sensitivity, Ben Biochemical Enterprise, Milan, Italy): samples with UPs >250 mg/dL were manually diluted 1:5 with distilled water and re-analysed to avoid inaccurate measurement for values outside the linearity of the method. The UC was measured with a modified Jaffe method (Real Time Diagnostic Systems, Viterbo, Italy). Samples were manually diluted 1:20 with distilled water to fit the linearity of the method (Rossi et al., 2012).

The UPC ratio and the GGT/UC ratio were then calculated

Statistical analysis

Results regarding the ~~the~~ GGT/UC ratio recorded in sequential samplings were compared to each other using a non parametric ANOVA for paired data (Friedmann test), followed by a Bonferroni test to compare the results recorded after treatment with the baseline values (i.e. the values recorded at admission, before any treatment). Statistical analyses were run in a specific software (Analyse-it version 2.21, Analyse-it Software Ltd, Leeds, UK) and the level of significance was set at ~~P~~*p*<0.05

Results

All the dogs enrolled in the study remained in IRIS stage I during the study period and their clinical condition improved in 1-3 weeks of treatment.

Results regarding urinary findings are reported in table 1.

Compared with values recorded at admission, at the end of the study period the UPC ratio decreased in 5/10 dogs, and increased in 5/10 dogs despite the amelioration of clinical signs. In 3 out of these latter 5 dogs the increase was severe (the UPC values increased more than 2 times). Three out of the

nine proteinuric dogs became borderline proteinuric at the end of the study period while the borderline proteinuric dog became frankly proteinuric. Due to this variable behavior, no significant differences were found between the UPC values of sequential samplings (figure 1).

Compared with the pre-treatment sample, at the end of the study period the GGT/CU ratio decreased in 8/10, remained substantially unchanged in 1/10 dogs and increased in 1/10 dogs. Moreover, in 3/10 dogs values at the end of the study period the GGT/UC ratio decreased below the threshold consistent with tubular proteinuria (0.81) and in one out of these 3 dogs the decrease below this threshold occurred very early, at the second week of treatment. On a statistical point of view, values at 6 weeks were significantly lower than before treatment (figure 1).

However, transient increases of both the UPC and the GGT/UC ratio were found also in those cases on which the value at the end of the study period finally decreased.

Discussion

The early phase of canine leishmaniasis is characterized by the progressive development of an immune-mediated glomerulopathy that induces a proteinuric nephropathy. The passage of proteins through the glomerular barrier may induce functional tubular impairment, that may theoretically be restored when the overflow of proteins decreases after anti-leishmaniotic treatment, or structural damages of tubular cells, that may persist despite the improvement of clinical signs after treatments. Hence, this study was designed to assess whether in leishmaniotic dogs with changes consistent with tubular proteinuria at first visit that showed an amelioration of clinical signs after conventional treatments, the simultaneous evaluation of the UPC ratio and the GGT/UC may provide additional information, compared with the recommended approach (Roura et al., 2013), that includes only the evaluation of the UPC. The addition of the GGT/UC may in fact differentiate dogs with transient impairment of tubular functions from those with permanent tubular damages.

The GGT/UC has been chosen as an alternative method to investigate the presence of tubular damage since GGT may be released from the membranes of damaged tubular cells (Brunker et al., 2009) and

its measurement in urine is less invasive then renal biopsy, that is the gold standard for assessing tubular damage (IRIS Canine GN Study Group, Diagnosis Subgroup, 2013), and is cheaper and easier to perform in house in routine practice than SDS electrophoretic techniques, that well correlate with results of renal biopsies (Brown et al., 2010; Zini et al., 2004) or than analytes that have been more recently proposed for the investigation of renal function such as retinol binding protein (RBP) or Neutrophil gelatinase-associated lipocalin (NGAL) (Hokamp et al., 2016) .

Since storage, either at 4°C or at -20°C, may inactivate the enzyme and provide false negative results (Flandrois et al., 1989), only samples on which the activity of GGT was measured soon after sampling and centrifugation were included in the study.

The results of this study demonstrated that both the markers showed transiently increased during the study period, as already demonstrated in previous studies on proteinuria (Pierantozzi et al., 2013; Plevraki et al., 2006), likely depending on the release of antigens after the death of the parasite, with subsequent temporary worsening or immune-complex glomerulonephritis, that induces the increase of the UPC, and a subsequent overflow of proteins that it may be responsible for a transient functional impairment of tubular functions.

Apart from these fluctuations, the UPC decreased, at the end of the study period, only in half of the dogs, on which, however, values remained in the proteinuric or borderline proteinuric range, and no significant differences compared with values recorded at admission were found. This is not surprising, since significant differences of the UPC may require up to 8 weeks after treatment (Pierantozzi et al.,, 2013), and in most cases dogs were still proteinuric also in previous longitudinal studies (Pierantozzi et al., 2013; Plevraki et al., 2006), likely because the immune-mediated glomerulonephritis that characterizes canine leishmaniasis may induce permanent and non reversible glomerular damage (Koutinas and Koutinas, 2014)

Conversely, the GGT/UC decreased in the large majority of dogs, its decrease was statistically significant, and values decreased below the threshold consistent with tubular damage in 3 dogs, suggesting that in most cases the tubular proteins detectable at admission were likely due to a

functional impairment of tubular cells due to the overflow of proteins associated with glomerular damages and that this impairment disappear when the overflow of proteins decreases after treatment. This seems to contrast with a previous study on which no significant differences in the GGT/UC were found after 4 weeks of treatment compared with the pre-treatment levels (Pardo-Marin et al., 2017). However, in the cited study urinary GGT was measured in frozen urine and results may therefore have been biased by the storage artifacts mentioned above (Flandrois et al., 1989). This may explain why in the former study the GGT/UC did not decrease after treatment as the other tubular markers included in the study. Conversely, in the current study only in a minority of dogs the GGT/CU did not change at the end of the study period compared with pre-treatment values. This suggests that in a few dogs the tubulointerstitial damage persists after treatment, as evidenced in some studies based on histopathology (Aresu et al., 2013).

This study has two main limitations: one is the low number of cases, that may reduce the statistical relevance of the results, due to the difficulties to obtain the whole series of samples during the follow up, in turn depending on a poor compliance of the owners. However, this allowed us to perform the study on a population with standardized inclusion criteria and time samplings. The second limitation is the lack of histopathologic findings, that cannot be repeatedly performed in field conditions for obvious ethical reasons,

In conclusion, this study demonstrates that the coupled analysis of both the UPC and the GGT/UC ratio may provide additional information compared with the simple analysis of the UPC since this latter may remain in the proteinuric range due to the persistency of glomerular lesions, while the normalization of the GGT/UC may differentiate the dogs that at the first visit had a functional impairment of tubular cells, from those on which tubulointerstitial damages persist despite the normalization of clinical signs, on which the GGT/UC remain high. Despite the limitations mentioned above, these results are encouraging to design future studies, eventually based on longer observation times or on the comparison with the results of renal biopsies. Moreover, it would be interesting in the

future to assess whether the non invasive technique investigated in the current study may provide relevant clinical information on the management of dogs that do not respond to treatments.

Acknowledgments

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Conflict of interest

The Authors do not have any conflict of interest potentially interfering with the results of this study

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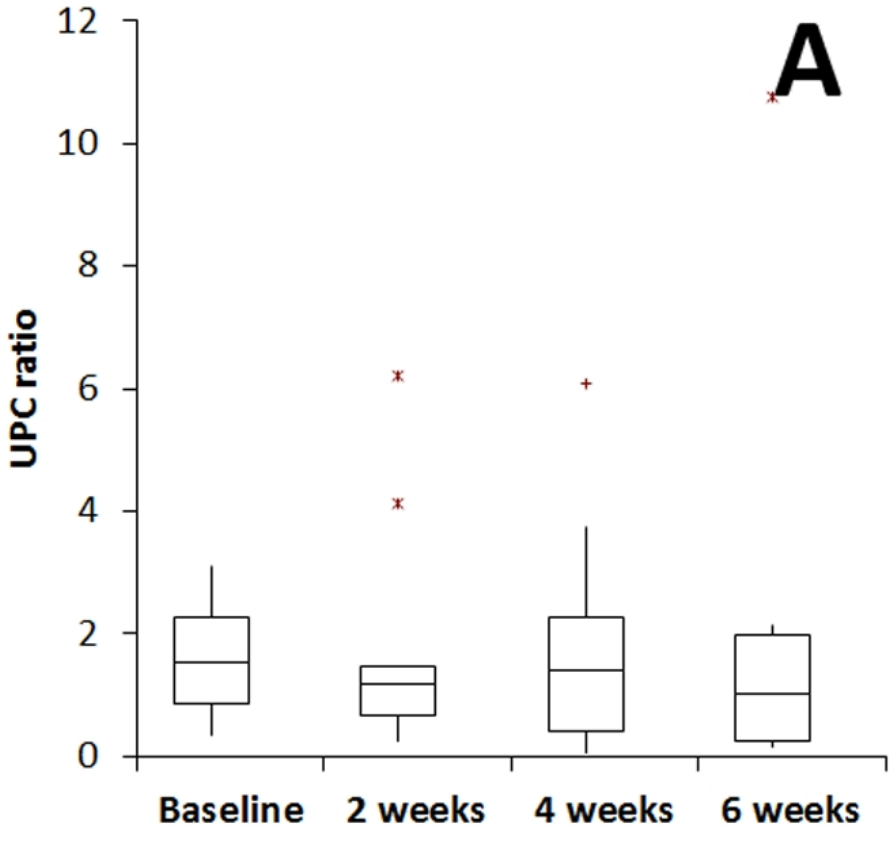
Figure captions

Figure 1: Data distribution and statistical analysis of results regarding the UPC ratio (A) and the GGT/UC ratio (B) recorded over time. The boxes indicate the I–III interquartile interval, the horizontal line corresponds to the median, vertical lines are the limits of outlier distribution according to the Tukey rule. Near outliers are indicated by the symbols "x" and far outliers with asterisks outside the boxes. The bolded asterisk within the boxes indicates a statistical difference (p~~P~~<0.05) compared with the baseline value


A
UPC ratio
12
10
8
6
4
2
0
Baseline
2 weeks
4 weeks
6 weeks

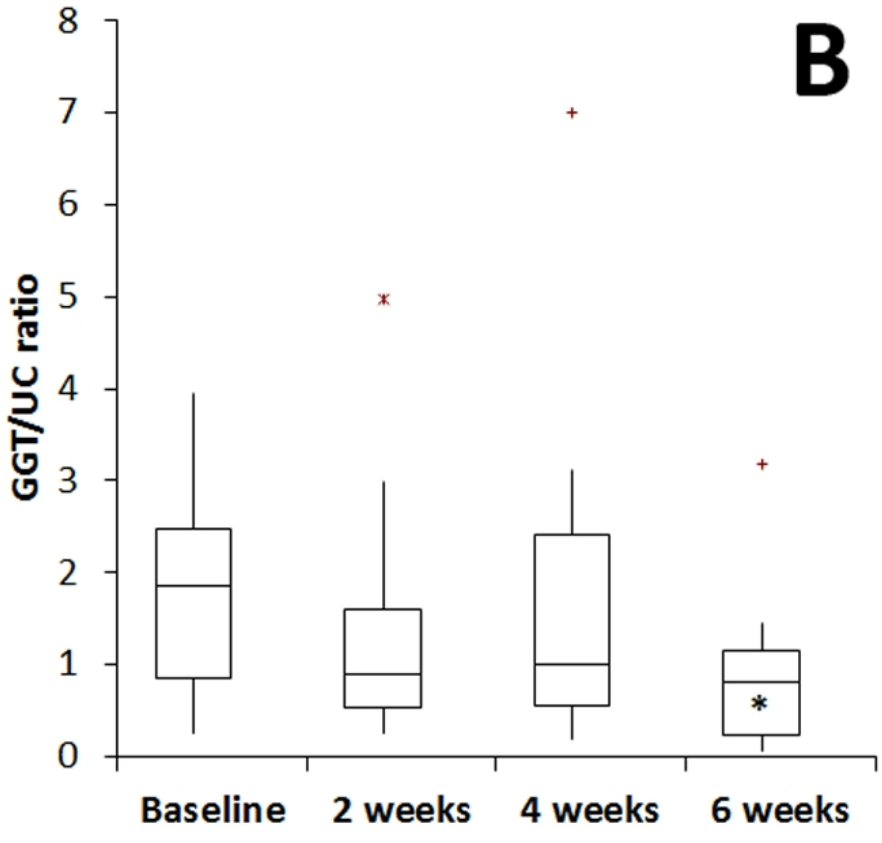

B
GGT/UC ratio
8
7
6
5
4
3
2
1
0
Baseline
2 weeks
4 weeks
6 weeks

Table 1: Results recorded in the all the samples included in this study

Dog n	Before treatment			2 weeks			4 weeks			6 weeks		
	UPC	IRIS	GGT	UPC	IRIS	GGT	UPC	IRIS	GGT	UPC	IRIS	GGT
1	1.52	P	3.3	0.25	BP	1.42	0.27	BP	1.81	0.28	BP	0.94
2	1.04	P	1.04	0.79	P	0.89	2.30	P	2.54	1.36	P	1.17
3	1.45	P	2.48	1.31	P	2.99	1.23	P	7.01	0.76	P	3.18
4	1.57	P	3.94	0.81	P	4.97	0.35	BP	3.11	2.13	P	0.81
5	0.84	P	1.85	1.42	P	1.23	0.79	P	0.91	1.03	P	1.11
6	0.36	BP	0.85	0.63	P	0.75	1.74	P	1.01	2.10	P	0.69
7	2.36	P	0.91	1.18	P	0.49	0.05	NP	0.28	0.23	BP	0.15
8	1.89	P	0.81	1.47	P	0.77	1.40	P	0.52	1.21	P	0.61
9	3.11	P	2.15	0.34	BP	1.63	3.73	P	1.06	10.76	P	1.45
10	3.03	P	2.46	4.14	P	0.35	6.08	P	0.69	0.23	BP	0.09

BP = borderline proteinuric (UPC ratio between 0.2 and 0.5); GGT/CU = γ-glutamyl transferase/urinary creatinine ratio; IRIS = staging of proteinuria according to the International Renal Interest Society (IRIS); NP = non proteinuric (UPC ratio<0.2); P = proteinuric (UPC ratio >0.50); UPC = urinary protein to creatinine ratio