Monitoring the response of canine hyperadrenocorticism to trilostane treatment by assessment of acute phase protein concentrations

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Abstract.

Background: Acute phase proteins (APPS) include haptoglobin (Hp), C-reactive protein (CRP) and serum amyloid-A (SAA). Increased Hp concentrations may be induced by endogenous or exogenous glucocorticoids in dogs. **Objectives:** To assess whether control of HAC affects the concentrations of Hp, CRP, SAA, alkaline phosphatase (ALKP) and cholesterol, to determine whether these analytes can be used to assess control of HAC following trilostane treatment, and whether a combination of these tests offers a valid method of assessing disease control. **Methods:** Hp, CRP, SAA, ALKP and cholesterol were assessed in 11 dogs with spontaneous HAC before and after treatment with trilostane. Adequate control of HAC was defined as post ACTH cortisol <150 nmol/l. **Results:** Significant reductions in Hp, ALKP, cholesterol and SAA (p<.05) but not of CRP were found after control of HAC. Only Hp, Cholesterol and ALKP were moderately informative (*Se* & *Sp*>0.7) of disease control when compared to ACTH stimulation test. SAA and CRP were unhelpful (*Se* & *Sp*<0.7). The analysis of the combination of the analytes did not improve the correlation with ACTH stimulation test. **Clinical relevance:** Relying on these analytes does not provide additional information over ACTH stimulation test results when assessing control of HAC treated with trilostane.

Key words: Acute phase proteins, alkaline phosphatase, canine, hyperadrenocorticism, trilostane.

Introduction

Following injury, cytokines induce changes in the concentrations of some glycoproteins (acute phase proteins –APPS) synthesised primarily by the liver, APPS

include haptoglobin (Hp), C-reactive protein (CRP), serum amyloid-A (SAA), cerulopasmin, α_1-acid glycoprotein and fibrinogen (Ceron and others 2005). The pattern of APPS concentration varies with the species and nature of the injury (Eckersall and others 1999). APPS are considered a useful tool for diagnosis, prognosis and monitoring response to treatment in human medicine (Child and others 1978, Kushner and Mackiewicz 1987, Thomson and others 1992). The availability of validated commercial veterinary kits has increased their use in non-human species.

Hyperadrenocorticism (HAC) is a commonly diagnosed canine endocrinopathy (Reusch and Feldman 1991). Trilostane (Vetoryl, Dechra Veterinary Products Ltd, Shrewsbury, UK), is currently, the only licensed drug for use in dogs with HAC in the UK. Trilostane a reversible competitive inhibitor of 3β-hydroxysteroid dehydrogenase blocks steroid biosynthesis in the adrenal gland, thereby inhibiting cortisol production. The ACTH stimulation test is currently recommended to monitor HAC treatment (Neiger and others 2002, Ruckstuhl and others 2002, Herrtage 2004).

Serum Hp concentrations are increased by endogenous and exogenous glucocorticoids in dogs (Harvey and West 1987, Martinez-Subiela and others 2004). This has been attributed to direct steroid induction (McGrotty and others 2003). Exogenous glucocorticoids do not affect the concentrations of other APPS such as CRP and SAA (Thomson and others 1992). Changes in CRP and SAA in dogs with hyperadrenocorticism have not been previously reported. We have previously shown that Hp is increased in dogs with HAC whilst dogs that have been treated for HAC have lower (though still increased) concentrations of Hp (McGrotty and others 2005).

Serum alkaline phosphatase (ALKP) activity and cholesterol are the most consistently increased biochemical parameters reported in dogs with uncontrolled HAC (76% and 90% of the cases respectively) (Ling and others 1979). Both these parameters have been shown to decrease significantly following treatment (Ruckstuhl and others 2002, Perez-Alenza and others 2006).

Urine cortisol to creatinine ratio and low dose dexamethasone suppression test (LDDST) are not useful for monitoring disease control following therapy (Angles and others 1997, Ruckstuhl and others 2002, Braddock and others 2003). Alternative tests are required because of the expense and availability of synthetic ACTH in certain countries (Behrend and others 2006). Even in those countries where ACTH is relatively inexpensive, ACTH stimulation tests do not assess the long term control of cortisol. Long term control of cortisol is required if HAC is to be successfully managed. For this reason, a marker that reflects chronic cortisol control (similar to fructosamine in diabetic patients) would be valuable.

The aim of this study was to assess whether control of hyperadrenocorticism by trilostane therapy (defined by post ACTH serum cortisol concentrations) significantly affected the serum concentration of APPS (Hp, CRP and SAA), ALKP, and cholesterol. The secondary aim was to determine whether APPS, ALKP and cholesterol could provide an alternative method of assessing control of canine HAC treated with trilostane and finally if a combination of these tests analytes offered a better validity in assessing disease control.

Materials and methods

Sixteen client-owned dogs were included in the study. All dogs had clinical signs, physical examination findings, routine biochemistry and haematology results consistent with HAC (Herrtage 2004). The diagnosis was confirmed by an intravenous ACTH (Synacthen, Alliance Pharmaceuticals Ltd, Whiltshire, UK) stimulation test and/or failure to suppress cortisol levels following intravenous administration of low dose dexamethasone and evidence of unilateral or bilateral adrenal gland enlargement on abdominal ultrasound. ACTH stimulation and LDDS tests were performed as previously described (Herrtage 2004). Ethical approval for all procedures performed on these cases was obtained from a local ethics committee acting under guidance from the UK Home Office.

All the analytes were assessed in samples taken before ACTH administration (with the exception of post ACTH cortisol) at initial presentation and again at 2, 4, 12 and 24 weeks after initiating trilostane therapy (starting dose of 30-60 mg PO q 12-24 h). For the purposes of this study, control of HAC was defined as a post-ACTH cortisol concentration below 150 nmol/l (Herrtage 2004, McGrotty and others 2005), with the test being performed four to six hours after trilostane administration. The analytes were recorded at the first time point when control was achieved and these results were then compared to pre-treatment values. Dogs were excluded from the study if control of HAC was not achieved. Dogs with adrenal dependant hyperadrenocorticism where also removed from the statistical analysis. None of the dogs were receiving any other drugs during the study.

Serum for APPS assessment was collected during routine jugular venipuncture and frozen at-20° C for batch analysis at a later date. Haptoglobin was measured using a

method previously reported (McGrotty and others 2003). CRP and SAA were measured using a microtitre plate reader (Tridelta Development Ltd, Ireland) designed for use in determining SAA concentrations in various animal species and validated for canine serum samples in our laboratory. The precisions of the assays were previously assessed by calculation of the intra- and inter-assay coefficients of variation (CV). The intra-assay CV was assessed by calculating the CV between duplicates (Fraser 1986), and was found to be 1.82% and 2.85% per cent over duplicate pairs over a Hp range of 0.29 to 0.72 g/l, 1.0 % and 2.8 % per cent over duplicate pairs over a CRP range of 18 to 74 µg/ml, and 3 % and 1.2 % per cent over duplicate pairs over a SAA range of 46.7 to 178 µg/ml. The inter-assay variation was also calculated based on replicates of control samples on two occasions. The CVs were 5.63 % and 4.83 % with mean Hp concentrations of 0.28 g/l and 0.73 g/l. For CRP and SAA the inter-assay variations were calculated based on control samples assayed in each assay performed. The CVs were 11.1 % and 12.6 % with mean CRP concentrations of 19 µg/ml and 75 µg/ml (Mishcke and others 2007), and 26 % and 15 % with mean SAA concentrations of 56 µg/ml and 189 µg/ml (ReactivLab, University of Glasgow, Bearsden, Scotland, data on file).

Accuracy was confirmed with serial dilutions between standards and dilutions of serum from dogs with raised CRP and SAA concentrations. The reference range for canine Hp using this assay has been previously reported as 0 to 2.2 g/l, while concentrations above 10 g/l are considered evidence of a major inflammatory response (Eckersall and others 1999a). The reference range for canine CRP is 0.46-9.6 µg/ml (Mischke and others 2007). The reference range for SAA is 0.08 to 8.75ug/ml (ReactivLab, data on file).

159 Plasma alkaline phosphatase was measured using a standard assay in a commercial
160 laboratory (Nationwide Laboratories, Lancashire, UK). The reference ranges for
161 canine ALKP (0-100 IU/l) and cholesterol (3.9-7.8 mmol/l) used in this study were
162 provided by the laboratory. Serum cortisol concentrations before and after ACTH
163 stimulation where measured using commercially available solid phase
164 radioimmunoassay kits (Coat-a-Count, DPC) previously validated for use in dogs.
165 (Cambridge Specialist Laboratory Services Ltd, Cambridge, UK).
166
167 Statistical analyses were conducted using SAS statistical software (release 9.1, ©
168 2002-03, SAS Institute Inc., Cary, NC, USA). A Wilcoxon signed rank test was used
169 to assess change in the analytes concentration at first presentation (time = 0)
170 compared to disease control (time =1). This non parametric test was preferred to the
171 corresponding parametric paired t-test because distributions of differences (significant
172 difference $P < .05$) in metabolite concentration showed distribution unlikely to be
173 normal, an important assumption of the parametric test.
174
175 Receiver-operating characteristic (ROC) curves for Hp, SAA, CRP, ALKP and
176 cholesterol were plotted using an on-line SAS macro, %ROCPLOT
177 (http://support.sas.com/kb/25/018.html) to assess for adequate specificity and
178 sensitivity in the assessment of disease control at various cut-off values of analytes.
179 Another on-line macro, %ROC (http://support.sas.com/kb/25/017.html) was used for
180 calculation of areas under ROC curves and their confidence limits.
181
182 Different analyte combinations were then tested in series or parallel after determining
183 their covariance (Dohoo and others 2003). Sensitivity (*Se*) and specificity (*Sp*) at the

optimal cut off values (maximum *Se* and *Sp*) for different analytes was determined and used to evaluate whether a pair of analytes used in series and/or parallel would have better discriminating ability.

Results

Sixteen dogs of various breeds with spontaneous HAC were included in this study. Dogs ranged from 6 to 13 years (mean 9.4, median 9.3). Eight were male and 8 female, weight range from 4.2 to 46 kg (mean 20.29, median 15). Eleven were diagnosed with pituitary dependant HAC and 3 with adrenal dependant HAC. Five dogs were removed from the study. Three were adrenal dependant, one did not achieve a post ACTH cortisol <150 nmol/l, and the other due to insufficient laboratory data. A post ACTH cortisol reduction <150 nmol/ with reduction of clinical signs was achieved in the remaining 14 dogs that were included in the final analysis. All dogs except one received trilostane twice daily. The target post ACTH cortisol <150 nmol/l occurred at week 2 in 6 dogs, at week 12 in 2 dogs and at week 24 in 3 dogs.

There was a statistically significant reduction in Hp, SAA, ALKP and cholesterol concentrations pre and post trilostane treatment. However, no statistically significant difference in pre and post treatment CRP values was found. Before treatment, 100% of dogs had Hp concentrations above the reference range and 9.09% (1/11) and 18.1% (2/11) had increased CRP and SAA serum concentrations respectively. After achieving control 100% (11/11), 18.1% (2/11) and 9.09% (1/11) had Hp, CRP and SAA concentrations above reference range respectively. All dogs both before and after treatment with trilostane had increased ALKP concentrations. Cholesterol

concentrations were increased in 90.9% of dogs (10/11) before and 45.45% (5/11) after trilostane treatment (Table 1).

Receiver-operating characteristic (ROC) curves were obtained (Figures 1a and 1b). Areas under the curves (AUC) and their 95% confidence limits (Table 2) indicate that the AUC for various analytes ranged from 0.58 to 0.82. *Se* and *Sp* at optimal cut off values determined from ROC curves (Table 2) were greater than 0.7 only for Hp, Cholesterol and ALKP; other analytes had either *Se* or *Sp* lower than 0.7. Therefore, only combination of Hp, Cholesterol and ALKP was evaluated in series and parallel. When they were tested in parallel, the combined *Se* was higher (0.95) but *Sp* was lower (0.55). In contrast, *Se* was lower (0.59) and *Sp* higher (0.93) when they were tested in series.

Discussion

This study showed a significant decrease in Hp values after trilostane treatment in dogs with HAC, although Hp remained above the reference range in all but one dog. This is in agreement with our previous study (McGrotty and others 2005). To the authors' knowledge this is the first report documenting CRP and SAA changes in dogs with naturally occurring HAC both pre and post trilostane treatment. Although we found a significant reduction in SAA concentration following control of HAC, this result has to be interpreted with caution as most dogs in this study had SAA within the reference range both before and after treatment. As may occur in Hp concentrations, increase of CRP in one of the dogs of our study following control of the HAC, may be associated with a concurrent or underlying inflammatory condition that was not

detectable during clinical examination (Onishi and others 2000, Kobelt and others 2003, Ceron and others 2005, Tecles and others 2005).

A variety of diseases have been associated with an increase in Hp (Harvey and West 1987, McGrotty and others 2003, Martinez-Subiela and others 2004). Concurrent inflammatory conditions reported in dogs with HAC, even after control of disease that could account for ongoing Hp elevation include pyoderma, urinary tract infection, osteoarthritis and neoplasia (Feldman and Nelson 2004). These conditions were not clinically apparent in the study dogs except pituitary or adrenal neoplasia. However subclinical disease cannot be excluded. Accumulation of endogenous ACTH and cortisol precursors occurs after trilostane treatment (Siebert-Ruckstuhl and others 2006). Dogs with atypical hyperadrenocorticism and increase blood levels of steroid hormones other than cortisol may have similar blood biochemical changes (Oliver 2007). Therefore the accumulation of cortisol precursor in dogs treated with trilostane may also contribute to the elevation of other analytes such as ALKP and Hp.

Meijer (1980) suggested that ALKP activity was one of the most useful routine laboratory tests in supporting clinical suspicion of HAC and previous studies have found a significant reduction of ALKP following trilostane therapy (Ruckstuhl and others 2002, Perez-Alenza and others 2006). In agreement with these reports, we found an elevated ALKP in all dogs prior to treatment and significant reduction in ALKP after treatment, but values remained above the reference range. Short duration of trilostane activity, enzymatic induction due to accumulation of other cortisol precursors or presence of concurrent disease processes (Neiger and Hurley 2001, Dunn and others 1995, Siebert-Ruckstuhl and others 2006) could account for this

finding. In most dogs with HAC, steroid induced isoform of ALKP (SIALKP) accounts for 70-90% of the total ALKP activity (Wilson and Feldman 1992). In the present study only total serum ALKP was assessed. Measuring SIALKP may have yielded more significant results. Although several studies have analysed SIALKP for the screening of dogs with HAC (Teske and others 1989, Wilson and Feldman 1992, Solter and others 1993), there are no previous studies considering the use of either ALKP or SIALKP as a screening tool to assess control of canine HAC with trilostane or mitotane treatment.

In common with ALKP, cholesterol has been shown to be increased in dogs with HAC (Ling and others 1979, Meijer 1980). In agreement with the findings of our study, significant reductions in serum cholesterol have been previously reported following control of HAC with trilostane (Ruckstuhl and others 2002). An improvement of the lipid enzymatic pathways, as a result of decrease cortisol may account for the reduction of cholesterol post treatment. The effect of increased endogenous ACTH and other cortisol precursors (Siebert-Ruckstuhl and others 2006) is unknown but may account for the ongoing elevation of cholesterol in some of the study dogs.

The second aim of this study was to determine whether APPS, ALKP and cholesterol concentrations could provide an alternative method of assessing control of canine HAC treated with trilostane. The ACTH stimulation test is currently recommended for the assessment of control of canine HAC treated either with mitotane (Dunn and others 1995) or trilostane (Neiger and others 2002, Ruckstuhl and others 2002, Braddock and others 2003). The range of post ACTH serum cortisol concentrations

in which control has been defined for dogs on trilostane varies from 30 to 250 nmol/l (Neiger and others 2002, Ruckstuhl and others 2002, Braddock and others 2003). We used an arbitrary post-ACTH cortisol concentration (<150 nmol/l) following a previous study from our group (McGrotty and others 2005).

An alternative test is considered accurate, compared with the "gold standard", when the AUC is 0.9-1 at a given cut-off point. AUC between 0.7-0.9 is considered only moderately informative (Greiner and others 2000). Using ROC curves, we found Hp, Cholesterol and ALKP to be the most useful tools to assess control of disease after trilostane treatment because their areas under the ROC curve were higher than the areas of CRP and SAA. When comparing the Hp, Cholesterol and ALKP concentrations to post-ACTH cortisol concentrations, the maximum sensitivities and specificities of around 73% were only moderately informative. CRP and SAA are poor predictors of disease control. This was not unexpected in the case of the CRP following the lack of significant variation of its concentrations following trilostane treatment.

The final aim of the study was to assess whether a combination of analytes offered a better validity in assessment control of HAC. Combination with CRP and SAA was not pursued as lower *Se* and *Sp* of these analytes was likely to further reduce the *Se* and *Sp* of the combination (Dohoo and others 2003). The combinations of Hp, Cholesterol and ALKP in parallel and series were not helpful in assessing control of HAC due to reduction in *Sp* and *Se* of the combined test, respectively.

There are a number of limitations of this study. The low number of cases may limit the power of the study but this does not impact on the results found to be significant. In other words, the differences detected are more likely to be real. A study with a higher number of cases would be required to further assess the non-significant findings of this study. Another limitation of this study is the use of a statistical method to assess adequacy of several analytes based on a gold standard test (ACTH stimulation test). Therefore when comparing the different analytes with post-ACTH cortisol results we assume a diagnostic adequacy of this test is 100% (Greiner and others 2000). However, the ACTH stimulation test is not entirely specific nor sensitive and assessment of disease control in dogs on trilostane still relays on concurrent judgement of the clinical evolution of the patient (Braddock and others 2003, Feldman and Nelson 2004). Some other tests for the diagnosis and assessment of control of HAC such as intramuscular ACTH stimulation test, salivary cortisol and UCCR following low dexamethasone suppression tests are currently under evaluation (Kobelt and others 2003, Vaessen and others 2004, Behrend and others 2006).

We used an arbitrary cut-off for serum cortisol concentration of <150 nmol/l (Herrtage 2004, McGrotty and others 2005) for control of HAC. This does not necessarily equate to full clinical control. Other authors have suggested lower post-ACTH cortisol concentrations (<70 nmol/l) for well-controlled cases (Ruckstuhl and others 2002). Using of a lower cortisol cut-off may have offered more significant variations in some of the analytes tested. A cut off of a post-ACTH cortisol concentration of less than 15 nmol/l) has been proposed as excessive control of HAC (Braddock and others 2003). These dogs are at risk of hypocortisolemia, and

334 trilostane dose reduction may be required. None of the dogs of the study had cortisol
335 values below this point.
336
337 Measurement of APPS, ALKP and cholesterol may be altered by hyperlipidaemia,
338 hyperbilirubinaemia, and/or haemolysis (Kaplan and Pesce 1996, Martinez-Subiela S,
339 Ceron 2005). However, no obvious changes were reported by the laboratory in the
340 analysed samples. Dogs with an adrenal tumour may have different APPS behaviour
341 due to the concurrent ongoing inflammatory response due to the tumour itself (Teske
342 and others 1989), therefore, to avoid the influence of this inflammatory response, they
343 were excluded from the statistical analysis. The number of cases with adrenal disease
344 in our study was too small to analyse this effect. Further studies in a larger cohort of
345 dogs with adrenal dependant hyperadrenocorticism treated with trilostane are needed
346 to assess its effect on APPS concentrations. We only evaluated APPS, ALKP and
347 cholesterol at one defined point of control, which may not explain the behaviour of
348 the different metabolites over a longer period of time. Futher studies to assess other
349 APPS such as α_1-acid glycoprotein, ceruloplasmin or α_1-antiprotease in dogs with
350 adrenal and pituitary dependant HAC at different stages of control could be useful.
351
352
353 In conclusion, the current study revealed significant changes in Hp, SAA, ALKP and
354 cholesterol concentrations but no significant difference in CRP after control of HAC
355 with trilostane. Compared with ACTH stimulation test in dogs with HAC on trilostane
356 treatment, the study analytes were less to only moderately informative even in
357 combination. Therefore, routine measurement of Hp, CRP, SAA, ALKP and

cholesterol cannot be recommended to assess control of pituitary dependant hyperadrenocorticsm in dogs on trilostane treatment.

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		Time 0				Time 1				
	Units	Mean	Median	IQR	Range	Mean	Median	IQR	Range	P value
Hp	g/l	8.09	7.45	5.7	2.8-13.6	4.55	4.20	2.30	1.5-8.1	0.0002*
CRP	µg/ml	4.69	1.58	1.15	0-27.5	6.04	2.05	3.76	0.32-41.5	0.9
SAA	µg/ml	2.04	1.10	1.95	0-9.5	1.55	0.30	1.08	0-14.9	0.03*
ALKP	IU/l	1010.15	830.00	470	113-4091	456.92	269.00	406.50	118-2068	0.002*
Chol	mmol/l	10.18	9.60	3.3	6.7-16.8	7.35	6.70	2.77	4.8-11.5	0.001*

Table 1: Concentrations of the different analytes at first presentation (time: 0) and at first point of control (time: 1). IQR† indicates interquartile range. Values marked with * indicate significant statistical difference ($P<.05$).

	AUC†	SE§	Confidence Limits	Cut off	Se	Sp
Hp	0.82	0.09	0.62, 1.00	4.80	0.73	0.91
CRP	0.51	0.13	0.25, 0.77	2.26	0.46	0.82
SAA	0.69	0.12	0.45, 0.93	0.19	0.64	0.82
ALKP	0.74	0.12	0.50, 0.97	531.00	0.80	0.73
Chol	0.82	0.09	0.64, 1.00	6.2	0.60	1.0

Table 2: Area under the curves (AUC†) and their 95% confidence limits for the different analytes. SE§ represents standard error.

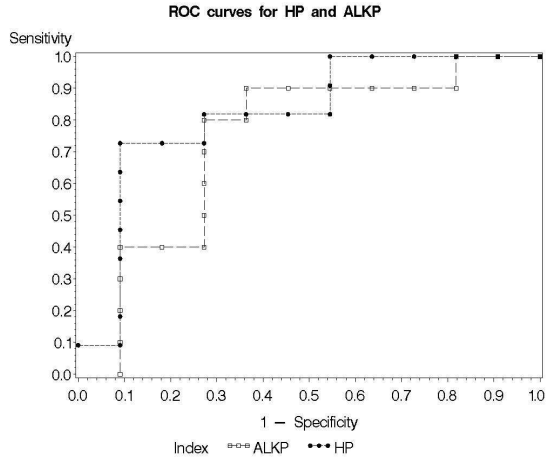

Fig 1a.

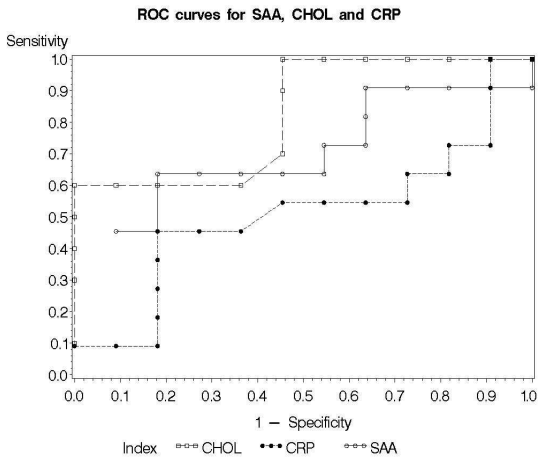

Fig 1b.

Figure 1a: Receiver operator characteristic (ROC) curve plots for haptoglobin (Hp) and alkaline phosphatase (alkp) after control of hyperadrenocorticism (cortisol post-ACTH< 150 nml/l).

Figure 1b: ROC curve plots for C-reactive protein (CRP), serum amyloid A (SAA), and cholesterol (Chol) after control of hyperadrenocorticism (cortisol post-ACTH< 150 nml/l).