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Effects of hydrocortisone on systemic arterial blood pressure and urinary protein excretion and "white coat effect" on blood pressure measurement in dogs

Schellenberg, Stefan

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vor, während und nach Hydrokortisongabe (n=6, I-HC, 8mg/kg bid für 90 Tage) oder Placebobehandlung (n=6) der Blutdruck, der Protein/Kreatinin-Quotient (UPC), die Mikroalbuminurie (MALB), der Urin-Albumin/Kreatinin-Quotient (UAC) bestimmt und eine Uringelektrophorese (SDS-AGE) durchgeführt. Während der Adaptationszeit sank der Blutdruck graduell und signifikant ab und erreichte nach 14 Tagen ein Plateau. Die medianen (Bereich) Werte der Blutdruckmessung mittels Dopplertechnik lagen bei 166 (149-200) mmHg initial, 145 (119-176) mmHg am Tag 9, 138 (118-165) mmHg am Tag 10, 127 (111-139) mmHg am Tag 35, 124 (115-143) mmHg am Tag 94 und 127 (114-142) mmHg am Tag 161. Im Vergleich zur initialen Blutdruckmessung, waren alle folgenden Blutdruckmessungen signifikant tiefer. Männliche Hunde zeigten zu jedem Zeitpunkt einen höheren Blutdruck als weibliche. Im zweiten Teil der Studie zeigte der Blutdruck und das UPC während der Hydrokortisongabe einen signifikanten Anstieg von 123 (114-136) mmHg respektive 0.17 (0.15-0.28) auf ein Maximum von 143 (128-148) mmHg und 0.38 (0.18-1.78) am Tag 28. Eine MALB entwickelte sich in 4 Hunden und der UAC zeigte bei allen Hunden einen signifikanten Anstieg mit einem Maximum am Tag 84. Der Blutdruckanstieg und die Proteinurie waren innerhalb von einem Monat nach Absetzen der Hydrocortisongabe vollständig reversibel. Die SDS-AGE zeigte, dass die Proteinurie hauptsächlich aus einer Albuminurie besteht mit einem deutlichen Anstieg während der Hydrokortisongabe. Des Weiteren wurde ein Protein mit einem Molekulargewicht von 25-30 kD im Urin von männlichen Hunden gefunden, das mittels Massenspektrometrie als Argininesterase identifiziert wurde, das wichtigste sekretorische Protein der Prostata. Zusammenfassend kann gesagt werden, dass Blutdruckmessungen bei Hunden, die nicht an die Blutdruckmessung gewöhnt sind falsch hoch sein können. Aus diesem Grund sollte bei klinischen Patienten ein erhöhter Blutdruck an mindestens 2 weiteren Tagen bestätigt werden, bevor die Diagnose einer Hypertension gestellt und eine Therapie begonnen wird. Eine Langzeit-Behandlung mit Hydrokortison führte zu einem signifikanten Blutdruckanstieg und einer erhöhten Proteinausscheidung über den Urin. Die Veränderungen waren nur leichtgradig und insbesondere der Blutdruck ist nicht auf ein hypertensives Niveau angestiegen. Alle Veränderungen waren innerhalb eines Monats nach Absetzen der Hydrokortisongabe reversibel.

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Klinik für Kleintiermedizin
der Vetsuisse-Fakultät Universität Zürich

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Arbeit unter der Leitung von Prof. Dr. Claudia Reusch und PD Dr. Tony Glaus

Effects of hydrocortisone on systemic arterial blood pressure and urinary protein excretion and „white coat effect“ on blood pressure measurement in dogs

Artikel 1: Effect of long-term adaptation on indirect measurements of systolic blood pressure in conscious untrained beagles

Artikel 2: The Effects of Hydrocortisone on Systemic Arterial Blood Pressure and Urinary Protein Excretion in Dogs

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
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vorgelegt von
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Abstract

Hypertension and proteinuria are commonly recognized in dogs with spontaneous hypercortisolism. There is, however, little information regarding the effect of exogenous glucocorticoids on blood pressure (BP) and proteinuria and whether these changes are reversible. Therefore the purpose of this study was to investigate if, how fast and to what degree experimental exogenous hypercortisolism results in hypertension and proteinuria, and whether these changes are reversible.

Stress or anxiety associated with unknown people, an unfamiliar environment or the circumstances of the measurement, can result in a considerable increase in an animal's BP. Therefore, in a first part, BP measurement was repeatedly performed in dogs over several weeks to investigate if and how much adaptation to the measurement procedure influences the results. In a second part, BP, urine protein:creatinine ratio (UPC), microalbuminuria (MALB), urine albumin:creatinine ratio (UAC) and urine gel electrophoresis were evaluated in 12 Beagle dogs before, during and after administration of hydrocortisone (n=6, I-HC, 8mg/kg PO bid for 90 days) or placebo (n=6).

During the adaptation period, the BP decreased gradually and significantly, and levelled out after 14 days. The median (range) of values obtained by Doppler were 166 (149 to 200) mmHg initially, 145 (119 to 176) mmHg on day 9, 138 (118 to 165) mmHg on day 10, 127 (111 to 139) mmHg on day 35, 124 (115 to 143) mmHg on day 94 and 127 (114 to 142) mmHg on day 161. All the later measurements were significantly lower than the initial measurement. Male dogs had higher BP than females on each occasion.

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In conclusion, measurements of blood pressure may be erroneously high in dogs not familiar with the measurement procedure. Therefore, in a clinical setting the measurement of an elevated blood pressure should be confirmed by repeating the measurement on at least two additional occasions before hypertension is diagnosed and antihypertensive treatment is started. Long-term hydrocortisone treatment, indeed, results in significant increases in systemic BP and urinary protein excretion. However, the changes are mild and in respect to BP, elevation to the point of systemic hypertension did not occur. Furthermore, all changes were reversible within one month after discontinuation of hydrocortisone.

Zusammenfassung

Der spontane Hyperkortisolismus beim Hund ist häufig mit Hypertension und Proteinurie assoziiert. Über den Effekt von exogenen Glukokortikoiden auf den Blutdruck, die Proteinurie und deren Reversibilität ist jedoch bislang nur wenig bekannt. Deshalb war das Ziel dieser Studie zu evaluieren ob, wie schnell und in welchem Masse experimentell-induzierter exogener Hyperkortisolismus zu Bluthochdruck und Proteinurie führt, und ob diese Veränderungen reversibel sind.

Stress oder Angst in Zusammenhang mit unbekanntem Menschen, einer ungewohnten Umgebung oder der Blutdruckmessung an sich, können zu einem erheblichen Blutdruckanstieg führen. Um den Einfluss einer Adaptation an die Blutdruckmessung zu untersuchen, wurde in einem ersten Teil der Studie der Blutdruck über mehrere Wochen wiederholt gemessen. In einem zweiten Teil wurden bei 12 Beagle Hunden vor, während und nach Hydrokortisongabe (n=6, I-HC, 8mg/kg bid für 90 Tage) oder Placebobehandlung (n=6) der Blutdruck, der Protein/Kreatinin-Quotient (UPC), die Mikroalbuminurie (MALB), der Urin-Albumin/Kreatinin-Quotient (UAC) bestimmt und eine Uringelektrophorese (SDS-AGE) durchgeführt.

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Zusammenfassend kann gesagt werden, dass Blutdruckmessungen bei Hunden, die nicht an die Blutdruckmessung gewöhnt sind falsch hoch sein können. Aus diesem Grund sollte bei klinischen Patienten ein erhöhter Blutdruck an mindestens 2 weiteren Tagen bestätigt werden, bevor die Diagnose einer Hypertension gestellt und eine Therapie begonnen wird. Eine Langzeit-Behandlung mit Hydrokortison führte zu einem signifikanten Blutdruckanstieg und einer erhöhten Proteinausscheidung über den Urin. Die Veränderungen waren nur leichtgradig und insbesondere der Blutdruck ist nicht auf ein hypertensives Niveau angestiegen. Alle Veränderungen waren innerhalb eines Monats nach Absetzen der Hydrokortisongabe reversibel.

Effect of long-term adaptation on indirect measurements of systolic blood pressure in conscious untrained beagles

S. SCHELLENBERG, T. M. GLAUS, C. E. REUSCH

To evaluate the effect of an adaptation period on systemic blood pressure readings, systolic blood pressure was measured in 12 young adult untrained beagles over several weeks by means of a Doppler flow detector and oscillometric devices. The pressure decreased gradually and significantly, and levelled out after 14 days. The median (range) of values obtained by Doppler were 166 (149 to 200) mmHg initially, 145 (119 to 176) mmHg on day 9, 138 (118 to 165) mmHg on day 10, 127 (111 to 139) mmHg on day 35, 124 (115 to 143) mmHg on day 94 and 127 (114 to 142) mmHg on day 161. All the later measurements were significantly lower than the initial measurement. Male dogs had higher blood pressures than females on each occasion. The blood pressure readings obtained with one of the oscillometric devices and the Doppler device were comparable and correlated significantly.

SYSTEMIC hypertension is a well recognised disorder in dogs, but in contrast to people, in whom primary 'essential' hypertension is most important (Kaplan 2001), hypertension in dogs most often appears to be secondary to an underlying disease, such as renal disease, hyperadrenocorticism, diabetes mellitus, pheochromocytoma, hyperaldosteronism and hypothyroidism (Breitschwerdt and others 1985, Gilson and others 1994, Bartges and others 1996, Ortega and others 1996, Struble and others 1998). A diagnosis of hypertension is based on the determination of systemic blood pressure. Although direct measurement is regarded as the gold standard (Kittleson and Olivier 1983), it is not usually feasible in practice and blood pressure is therefore measured by indirect methods. The Doppler flow detector and oscillometric devices have been validated in conscious dogs (Coulter and Keith 1984, Bodey and others 1996, Schneider 1999, Stepien and others 2003), but whichever device is used, blood pressure is a variable haemodynamic phenomenon that is influenced by many factors. Stress or anxiety associated with unknown people, an unfamiliar environment or the circumstances of the measurement, can result in a considerable increase in an animal's blood pressure (Belew and others 1999). This increase has been referred to as the white-coat effect and may lead to a false diagnosis of hypertension (Gosse and others 1993) and its unnecessary treatment. To minimise the effect it has been recommended that blood pressure should be measured in a quiet area and that the animal should be allowed to become acclimatised to its surroundings for at least five minutes. Furthermore, the first measurements should be discarded until the animal becomes accustomed to the procedure (Brown and Henik 1998). The importance of this short period of adaptation is well accepted, but little information is available about the long-term effects of adaptation. Remillard and others (1991) found that the diastolic, but not the systolic blood pressure of five healthy dogs decreased during a period of five weeks. The purpose of this study was therefore to evaluate the effect of long-term adaptation on indirect measurements of systolic blood pressure in conscious and untrained dogs.

MATERIALS AND METHODS

Twelve, three-year-old beagle dogs, six intact males and six intact females with bodyweights ranging from 10.4 to 16.6 kg (mean 13.2 kg) were used. They were considered to be healthy on the basis of the results of a physical examination, a complete blood count, a serum chemistry profile

and urinalysis. The dogs had been kept in their housing facility away from the veterinary hospital since birth and supervised by the same caretakers for several months before the study began; they had been used regularly for student education, including clinical examinations, palpation and auscultation.

Experimental protocol

The blood pressure of each dog was measured on 12 occasions, on days 0, 9, 10, 14, 16, 17, 35, 67, 94, 147, 154 and 161. On days 0, 9, 10, 35, 94 and 161 it was measured by the Doppler method; on the other six occasions it was measured with an oscillometric device; on days 14, 16 and 17 using the Memoprint (S + B medVET) and on days 67, 147 and 154 using the SDI Vet/BP 6000 (SDI). To minimise the effect of the natural diurnal variability of blood pressure (Gelzer and Ball 1997, Piccione and others 2005), the measurements were made at the same time of day in each dog.

All the measurements were made by one person (S. S.) in the dogs' housing facility, with either an oscillometric device or an ultrasonic Doppler device (Model 811-B; Parks Medical Electronics). The dogs were placed in left lateral recumbency and allowed to acclimatise to their surroundings for at least five minutes before any measurements were taken. The first readings were discarded, and the arithmetic mean of five consecutive measurements was used for the data analysis.

For the oscillometric measurements an inflatable cuff was placed directly around the base of the dog's tail without clipping the hair (Vincent and others 1993). A cuff width of approximately 40 per cent of the circumference of the tail was chosen (Edwards 1990).

The Doppler ultrasonographic measurements were made by using the techniques described by Edwards (1990), Crowe and Spreng (1995) and Spreng and others (1996). The Doppler ultrasound probe was fixed in position over the superficial palmar arterial arch after the hair had been clipped and coupling gel applied. A cuff width of approximately 40 per cent of its circumference was placed around the mid-antebrium. The cuff was inflated to 40 to 50 mmHg above the point at which the Doppler signal was no longer audible; the cuff was then slowly deflated and the systolic pressure was recorded as the pressure at which the pulse signal became audible.

Comparison of Doppler and oscillometric device measurements

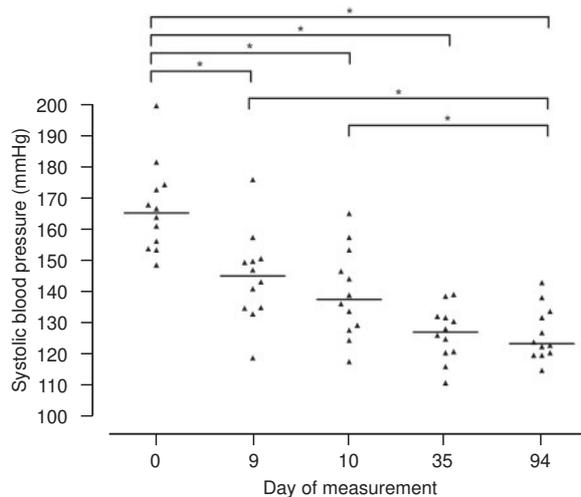
The measurements were made in the 12 dogs with the Doppler device and SDI Vet/BP 6000 oscillometric device, using the

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FIG 1: Median and individual measurements of systolic blood pressure made at intervals with the Doppler device in 12 conscious untrained beagles. *P<0.05



same procedure as described above except that 10 minutes of acclimatisation preceded each series of measurements. After acclimatisation 10 oscillometric readings from the tail followed by five Doppler measurements from the forearm were obtained. The measurements were repeated nine times over a period of six months, during which time the dogs' systolic blood pressures measured by Doppler ranged between 96 and 164 mmHg.

Statistical analyses

The results were analysed by non-parametric statistical methods using SPSS 11.0 for Windows (SPSS). Median values and ranges were reported. The change in blood pressure during the adaptation period was analysed by means of Friedman's repeated measures test and a post hoc Wilcoxon test. For multiple comparisons, a Bonferroni correction was applied to the Wilcoxon test. Differences between male and female dogs were compared by the Mann-Whitney U test. The coefficient of variation was used as an estimate of the variability of the results. The measurements made oscillometrically were compared with the Doppler measurements by regression analysis and correlation coefficient, using GraphPad

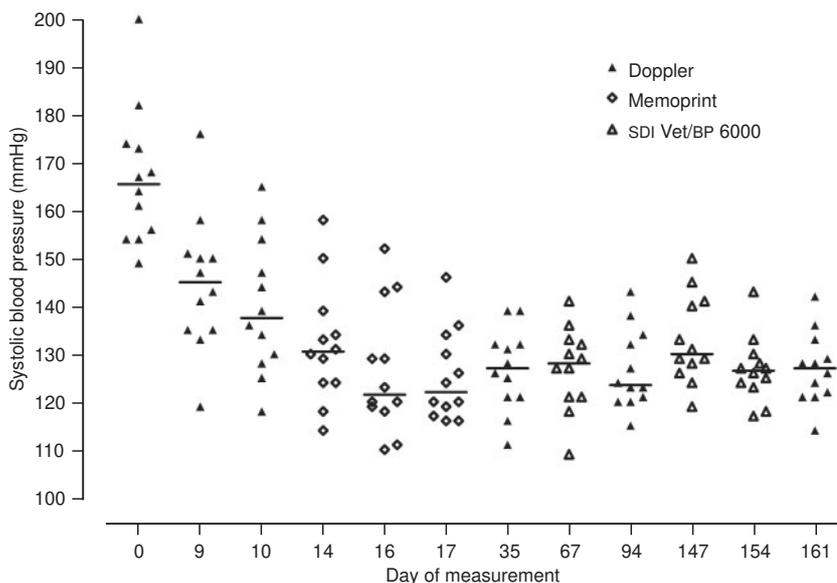


FIG 3: Measurements of systolic blood pressure made at intervals in 12 conscious, untrained beagles by Doppler, Memoprint and by SDI Vet/BP 6000

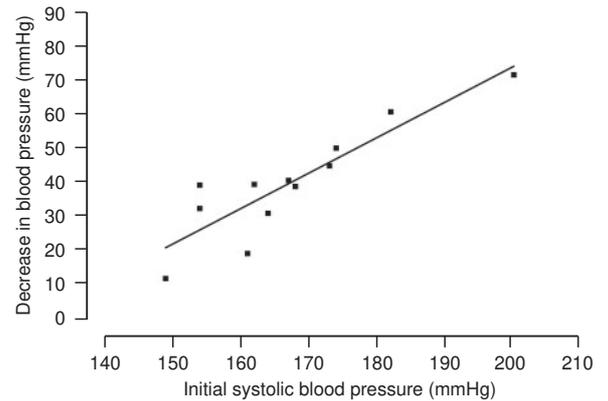


FIG 2: Relationship between the initial systolic blood pressure recorded in the 12 conscious, untrained beagles and the decrease observed after 161 days

Prism 4 (GraphPad Software). The results of the two methods were also analysed according to Bland and Altman (1986). Differences were considered significant at values of $P \leq 0.05$.

RESULTS

Effects of adaptation on blood pressure measured by Doppler

The systolic blood pressures measured by the Doppler method over a period of 94 days are shown in Fig 1. They showed a gradual and significant decrease over time. Initially eight of the 12 dogs had a systolic blood pressure of 161 mmHg or higher, but after nine days there had been a significant median decrease of 24 mmHg (range 4 to 40 mmHg) and there were further decreases after 10 and 35 days. After 94 days the pressure was significantly lower than after nine or 10 days. After the pressure had levelled out there was little variation in individual dogs from day to day, with a median coefficient of variation of 3.2 per cent (range 1.7 to 10.1 per cent). There was no significant difference between the mean blood pressure measured after 35, 94 and 161 days. The changes observed between the initial measurements and the measurements made 161 days later are shown in Fig 2. The dogs that initially had the highest systolic blood pressures had the largest decreases in blood pressure. The correlation coefficient of linear regression of the change in systolic blood pressures against the initial blood pressure was $r=0.891$ ($P<0.0001$).

There were initially wider variations in blood pressure between the dogs than there were later; initially, and after nine and 10 days the coefficients of variation were 8.9 per cent, 9.8 per cent and 10.1 per cent, respectively, compared with 6.7 per cent, 6.6 per cent and 5.8 per cent after 35, 94 and 161 days, respectively.

Effects of adaptation on the measurements

The measurements made with the three different devices are summarised in Fig 3. There was a steady reduction during the first 16 days, but thereafter the median systolic pressures were similar and fluctuated between 122 and 130 mmHg. During this period the measurements for each individual dog were reproducible with a coefficient of variation ranging from 1.9 to 8.0 per cent (median 4.2 per cent), although different methods of measurement were used.

Effects of sex

Throughout the study the male dogs had higher blood pressures than the females. The difference was not significant on individual days, but when all the measurements by the

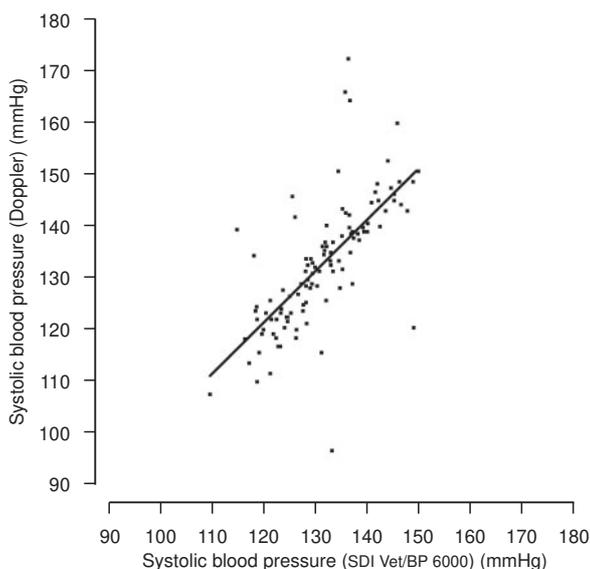


FIG 4: Relationship between the systolic blood pressures recorded in 12 conscious, untrained beagles by Doppler and by SDI Vet/BP 6000

Doppler method were compared, the male dogs had significantly higher pressures ($P=0.001$).

Comparison of Doppler and oscillometric device measurements

A set of 108 measurements with the Doppler and the SDI Vet/BP 6000 were analysed. Linear regression revealed a significant correlation ($P<0.0001$) with a slope of 1.007 between the two methods (Fig 4). The same data, analysed by the method of Bland and Altman (1986) are shown in Fig 5. The oscillometric device bias was -1.6 mmHg, and the limits of agreement were ± 17.8 mmHg; 69.4 per cent of the oscillometric device measurements were within 5 mmHg of the Doppler measurements and 88 per cent were within 10 mmHg.

DISCUSSION

For the diagnosis and treatment of systemic hypertension accurate and reliable blood pressure measurements are required. In people and dogs, it is well known that the act of measuring blood pressure leads to a rise in the systolic and diastolic blood pressure (Mancia and others 1983, Kallet and others 1997). This 'white-coat' effect develops at the beginning of a clinical evaluation and typically normalises within a few minutes (Mancia and others 1983). As a result, a short period of acclimatisation for five to 10 minutes is recommended to avoid the over-diagnosis and over-treatment of hypertension.

The results of this study show that in normotensive dogs previously unaccustomed to blood pressure measurements, their blood pressure was overestimated on the first few days and decreased significantly with repeated measurements on subsequent days. On the basis of their initial measurements eight of the 12 dogs satisfied conventional criteria for the diagnosis of hypertension, that is, a systolic pressure above 160 mmHg (Stepien and others 2003). However, on the second and third evaluation, only one dog, and on the following evaluations, no dogs, would have been classified as hypertensive. An erroneous diagnosis of hypertension would have been made despite applying the recommended acclimatisation period. Similar findings have been reported in human patients, whose blood pressure decreased after repeated measurements (Sinaiko and others 1989, Singh and others

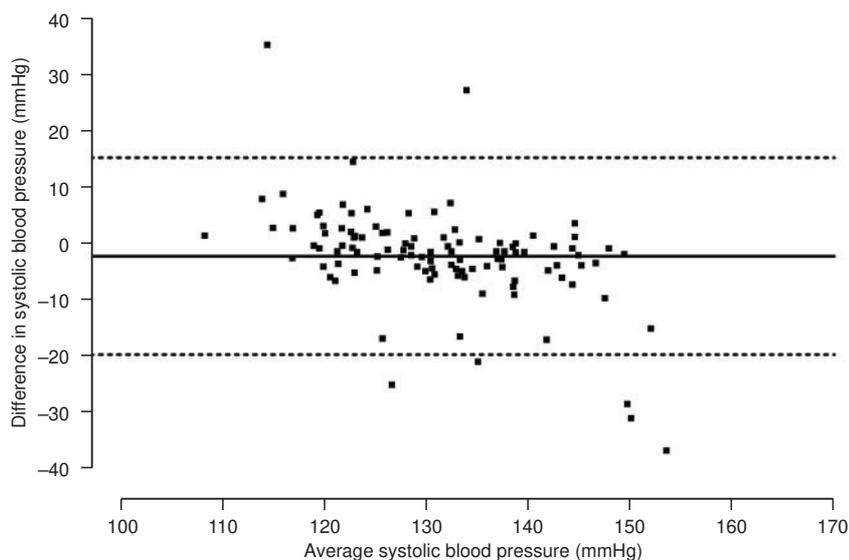


FIG 5: Bland-Altman plot of the difference between the measurements of systolic blood pressure in 12 conscious, untrained beagles made with SDI Vet/BP 6000 and the Doppler device and the average of their mean values. Solid line Mean difference, Dotted lines ± 2 standard deviations

1991). For this reason, in human medicine, at least three sets of readings at least a week apart should be obtained for a diagnosis of hypertension. If possible, the readings should be taken at home under varying conditions and at various times for at least four to six weeks with a semiautomatic device. If the diagnosis must be established more rapidly, a set of readings obtained with an automatic monitor over a period of 24 hours should be adequate (Kaplan 2001). In this study the dogs' blood pressure stabilised in a normotensive range between the fourth and fifth measurements, implying the importance of repeated measurements for a diagnosis of hypertension in dogs. After the dogs had become accustomed to the measurement procedure, their blood pressure did not change significantly even if the measurements were made after a long period of time.

The male dogs had a higher median blood pressure than females, in agreement with observations by Body and Michell (1996) in which entire male dogs had a higher blood pressure than neutered animals, and entire females had the lowest pressures. The reason is not understood, but thought to be due to a difference in temperament and a difference in sensitivity to stress.

This study was limited by not using the same method for measuring blood pressure on each occasion. The most pronounced decreases in pressure were observed at the beginning of the study, when only the Doppler device was used, but the comparison of the oscillometric and Doppler devices indicates that they give comparable readings under normotensive and mildly hypertensive conditions. The comparison of the Doppler and the Memoprint showed that there was good agreement between values in the normal range (110 to 160 mmHg), but only moderate or poor agreement in the hypotonic and hypertonic ranges (Schmiege 2002). During the study there were some outliers, with differences of up to 36 mmHg between the two methods, sometimes in the same dog (around 160 mmHg by Doppler and 130 mmHg by oscillometric device). These differences may have been due to real differences, because the measurements were not made simultaneously. It has been shown in dogs and people that large pressure fluctuations can occur, even within 60 seconds (Bovee 1993, Lossius and others 1993). The differences may also be associated with the different sites used to place the cuffs. However, there is always the potential for error when

a blood pressure measurement is obtained in a single individual, and the diagnosis of hypertension should always be made in relation to the clinical signs and any potential underlying disease.

Using research beagles instead of client-owned dogs may be another limitation of this study. Research beagles may be more nervous and excitable than other breeds, and may be less used to contact with different people and to manipulations. However, the beagles in this study had been living in their wards since birth, and had been handled by veterinarians for non-invasive procedures in the past. Thus, in some respects they may be considered to have been better adapted to veterinary manipulation than a client-owned dog presented to a clinic.

The results of this study indicate that measurements of blood pressure may be erroneously high in dogs not familiar with the measurement procedure. Repeated measurements on different days were associated with a significant decrease in pressure, and the decrease was greater when the initial pressures were higher. In a clinical setting blood pressure measurements should therefore be repeated on several days before hypertension is diagnosed and antihypertensive treatment is started.

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The effects of hydrocortisone on systemic arterial blood pressure and urinary protein excretion in dogs

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1 THE EFFECTS OF HYDROCORTISONE ON SYSTEMIC ARTERIAL BLOOD PRESSURE
2 AND URINARY PROTEIN EXCRETION IN DOGS

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11

12 Short title: Blood Pressure and Proteinuria in Hypercortisolism

13

14 An abstract of this study has been presented at the 2006 ACVIM Forum in Louisville, KY (J Vet
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24 ABSTRACT

25 Background: Hypertension and proteinuria are commonly recognized in dogs with spontaneous
26 hypercortisolism. There is, however, little information regarding the effect of exogenous
27 glucocorticoids on blood pressure (BP) and proteinuria and whether these changes are reversible.

28 Hypothesis: Hydrocortisone administration increases systemic BP and urinary protein excretion,
29 and these effects are reversible after hydrocortisone withdrawal.

30 Animals: 6 control dogs and 6 dogs treated with hydrocortisone.

31 Methods: BP, urine protein:creatinine ratio (UPC), microalbuminuria (MALB), urine
32 albumin:creatinine ratio (UAC) and urine gel electrophoresis were evaluated before, during and
33 after administration of hydrocortisone (8mg/kg p.o. q12h for 12 weeks) or placebo.

34 Results: BP and UPC increased significantly during hydrocortisone administration from 123
35 mmHg (range 114-136 mmHg) and 0.17 (0.15-0.28) to a maximum of 143 mmHg (128-148
36 mmHg) and 0.38 (0.18-1.78), respectively on day 28. MALB developed in four dogs and UAC
37 significantly increased in all dogs during hydrocortisone administration with the maximum on
38 day 84 (0.133 (0.034-1.251)). Both, BP elevation and proteinuria were reversible and completely
39 resolved within one month after stopping hydrocortisone administration. SDS-AGE revealed the
40 proteinuria to be primarily albuminuria with a pronounced increase during hydrocortisone
41 treatment. Furthermore, a protein of 25-30 kDa was found in male dogs, identified by mass
42 spectrometry to be arginine esterase, the major secretory prostatic protein.

43 Conclusions and Clinical Importance: Long-term hydrocortisone treatment results in significant
44 but only mild increases in systemic BP and urinary protein excretion, which are both reversible
45 within one month after discontinuation of hydrocortisone.

46 Key words: Hypercortisolism, Hypertension, Proteinuria, Albuminuria, Arginine Esterase

47 INTRODUCTION

48 Hypertension is a frequent finding in humans and dogs with exogenous or endogenous
49 glucocorticoid excess. The prevalence of systemic hypertension in human patients with
50 spontaneous (endogenous) hypercortisolism (HC) has been reported to be approximately 80%.¹
51 In experimental studies in humans, glucocorticoids have been shown to increase systolic (SAP)
52 as well as diastolic (DAP) arterial blood pressure.^{2,3} Significant increases in SAP were identified
53 within 24 hours of cortisol administration, but iatrogenic glucocorticoid-induced hypertension
54 appears to be less severe than in the natural disease.² The prevalence of hypertension in dogs
55 with spontaneous HC is similar at 59-86%.^{4,5} When treating dogs with spontaneous HC,
56 adequate suppression of cortisol secretion results in decreasing blood pressure (BP), however,
57 hypertension is still common in the population of well controlled HC.⁴ In contrast, in
58 experimental dogs treated with hydrocortisone for several weeks, SAP was not significantly
59 higher compared to placebo treated dogs.⁶

60 Proteinuria is also commonly observed in dogs with HC. The incidence of pathological
61 proteinuria, defined as a urine protein:creatinine ratio (UPC) greater than 1.0, has been reported
62 to be 44 - 75%.^{4,7} Experimentally, longterm administration of prednisolone resulted in
63 proteinuria of glomerular origin.⁸ The reversibility of proteinuria in spontaneous HC is
64 controversial. Whereas Ortega et al. found a significant decrease in UPC in dogs with well-
65 controlled HC, Hurley and Vaden did not.^{4,7}

66 Much of the existing information about the effects of glucocorticoids on blood pressure and
67 proteinuria has been generated by experimental studies that used prednisolone for the induction
68 of proteinuria and hypertension. However, in spontaneous HC the endogenous glucocorticoid
69 hydrocortisone seems to be the major cause of clinical and laboratory abnormalities.⁹

70 In view of the paucity of knowledge on the effect of hydrocortisone on systemic BP, urinary
71 protein excretion, and particularly the reversibility of such changes in dogs, the purpose of this
72 study was to induce experimental HC using hydrocortisone as an attempt to most closely mimic
73 the natural disease. The specific goals then were first to assess the development and severity of
74 hypertension, second quantitating and characterizing the glucocorticoid induced proteinuria,
75 third to assess the association between urinary protein excretion and BP, and fourth, to study the
76 reversibility of these changes.

77

78 MATERIAL AND METHODS

79 This randomized, placebo-controlled study was approved by the Cantonal Veterinary Office
80 (Zurich, Canton of Zurich, Switzerland).

81 **Dogs**

82 The study was conducted using twelve 3.5-year old Beagle dogs, 6 intact males and 6 intact
83 females, with a body weight ranging from 10.4 to 16.6 kg (median 12.9 kg). The dogs were
84 determined to be healthy on the basis of physical examination, CBC, serum biochemistry profile,
85 urinalysis, urine culture, UPC ratio and indirect BP measurement.

86 **Study Design**

87 HC was induced by hydrocortisone, the synthetic glucocorticoid most similar to cortisol. The
88 dosage was chosen extrapolating from previous studies inducing I-HC in dogs.^{6,8} Dogs were
89 randomly allocated to two groups of 6 dogs. Dogs in the control group received a placebo gelatin
90 capsule PO q12h while dogs in the treatment group received hydrocortisone^a at a median dose of
91 8.5 mg/kg (range of 7.5-9.6 mg/kg) PO q 12h for 84 days (I-HC group). Dogs were examined
92 before (d0), and 1 (d1), 5 (d5), 28 (d28), 56 (d56), 84 (d84) days after starting treatment and 1

93 (d1p), 5 (d5p), 28 (d28p), 56 (d56p) and 84 (d84p) days after withdrawal of hydrocortisone and
94 placebo, respectively. Dogs were observed for typical cortisol-induced clinical abnormalities
95 including PU/PD and hair loss, but only subjectively, i.e. water intake and urine production were
96 not quantitated and it was not attempted to use a scoring system for the skin abnormalities. On
97 d0, d28, d56, d84, and d1p an ACTH stimulation test was performed in all dogs by obtaining
98 samples for determination of cortisol before and 1 hour after IM injection of 0.25 mg of synthetic
99 ACTH^b. Cortisol concentrations were determined by use of a previously validated
100 chemiluminescence method (ADVIA Centaur[®] System, Bayer (Schweiz) AG, Zurich,
101 Switzerland).¹⁰

102 **Blood pressure measurement**

103 Before the beginning of the study, dogs were acclimated to the blood pressure measurement
104 procedure on 12 different days, to minimize excitement and anxiety during the study.^c Systolic
105 (SAP), diastolic (DAP) and mean (MAP) arterial pressure were measured using an indirect
106 oscillometric device^d. An inflatable cuff of approximately 40% of the tail circumference was
107 placed directly around the base of the tail without clipping hair.¹¹ Before recording BP, dogs
108 were placed in left lateral recumbency and allowed to acclimate to the surroundings for at least
109 10 minutes and the first BP readings were discarded. For data analysis the arithmetic mean of 10
110 measurements was used.

111 **Urinary Samples**

112 Urine samples were collected from each dog by use of ultrasound-guided cystocentesis for
113 routine urinalysis, urine culture, UPC, and for assessing microalbuminuria, urine
114 albumin:creatinine ratio (UAC) and urine protein electrophoresis.

115 **Assessment of proteinuria**

116 **Urine protein:creatinine ratio**

117 Total urine protein levels were assayed on an autoanalyzer by an immunoturbidimetric method.
118 Urine creatinine concentrations were determined using a commercial autoanalyzer-based kinetic
119 Jaffe reaction and UPC ratios were calculated.

120 **Microalbuminuria and urine albumin:creatinine ratio**

121 Urine albumin concentration was determined by two different methods. Microalbuminuria
122 (MALB) was assayed semiquantitatively by means of the canine E.R.D.-Health Screen test^e
123 after urine samples were normalized to a urine-specific gravity of 1.010 to allow comparison
124 among samples. The amount is indicated as none (negative), small (+), moderate (++), and high
125 (+++). To quantitate albuminuria, a modified human immunoturbidimetric assay^f validated for
126 the dog was used as described previously¹², and UAC ratios were calculated. To assess
127 sensitivity and specificity of the MALB, 2 different UAC cut-offs were chosen, first the
128 mean+2SD obtained in our control dogs, second 0.03, the cut-off for pathologic
129 microalbuminuria in people.¹³

130 **Urine protein electrophoresis**

131 Urinary proteins were separated by sodium dodecyl sulfate agarose gel electrophoresis (SDS-
132 AGE) using the Hydragel proteinurie kit^g.¹⁴ Briefly, twenty microliters of a 10 g/L SDS solution
133 were added to 80 μ L of urine. Five microliters were loaded to each well (5 wells/gel) and
134 submitted to electrophoresis in an SDS-imidazole pH 7.0 buffer. After the gel was completely
135 dried at 80 °C for 20 min, it was immersed for 30 min in an aqueous staining solution (acid
136 violet) and destained in two successive aqueous baths before it was immersed in a glycerol
137 aqueous solution and dried at 80°C (15 min).

138 **Characterization of the male specific protein**

139 Gel protein bands were cut with a scalpel and repeatedly washed in 25 mM NH₄HCO₃ with
140 subsequent shrinking of the gel pieces through addition of 50 % acetonitrile in 25 mM
141 NH₄HCO₃. The proteins were digested overnight by adding 19 ng/μl sequencing grade modified
142 trypsin^h in 47mM Tris pH 9. Peptides were extracted from the gel by sonification in buffer A (2
143 % acetonitrile, 0.1 % trifluoroacetic acid). For liquid chromatography electrospray ionisation
144 tandem mass spectrometry (LC-ESI-MS/MS) analysis the protein supernatant was run over a
145 C18 column eluting the peptides at 600 nl/min in 45 min by using a gradient of 95 % buffer A to
146 60 % buffer B (80 % acetonitrile, 0.1 % trifluoroacetic acid). Peptides were ionized using electro
147 spray ionization and directly injected into the massspectrometer LCQ Deca XPⁱ recording their
148 mass-to-charge ratio. Following each full MS spectrum the two most intense peaks were
149 automatically selected for fragmentation of these peptides and recording of the MSMS signals
150 (fragments). All MSMS spectra were submitted to Mascot¹⁵ for identification of the proteins.

151 **Statistical analysis**

152 Results were analyzed by use of nonparametric statistical methods^{j,k}, and reported as median and
153 ranges. Differences within one group were tested by use of Friedman's repeated measures test
154 followed by Dunn's post-tests. Differences between the groups were tested by use of the Mann-
155 Whitney U test. Differences were considered significant at values of $p \leq 0.05$.

156

157 **RESULTS**

158 **Induction of HC**

159 In this model, long-term hydrocortisone administration effectively created hypercortisolism. All
160 dogs receiving hydrocortisone developed clinical signs (polyuria, polydipsia, no regrowth of
161 clipped hair within one month, and thinning of the ventral abdominal skin with prominent

162 subcutaneous veins), and laboratory abnormalities (stress leukogram, increased alkaline
163 phosphatase activity, isosthenuria) consistent with cortisol excess. During medication dogs in the
164 I-HC group had significantly higher baseline plasma cortisol concentrations than dogs in the
165 control group (e.g. d56: 3 hours post pill 30.7 mcg/dl (13.3-57.6) versus 0.5 mcg/dl (0.1-2.5)).
166 Finally, 36 hours after hydrocortisone withdrawal dogs in the I-HC group failed to respond to
167 ACTH stimulation; median plasma cortisol concentrations before and after ACTH administration
168 were 0.25 mcg/dl (0.1 – 1.4 mcg/dl) and 0.45 mcg/dl (0.1 - 2 mcg/dl), respectively.

169 **Blood pressure**

170 Initially, median SAP, DAP and MAP were 128 mmHg (122 - 142 mmHg), 63 mmHg (55 - 83
171 mmHg), and 90 mmHg (78 - 104 mmHg) in the control group and 123 mmHg (114 - 136
172 mmHg), 69 mmHg (53 - 78 mmHg), and 90 mmHg (81 - 101 mmHg) in the I-HC group,
173 respectively. Hydrocortisone but not placebo treatment resulted in a progressively increasing
174 SAP, DAP and MAP that peaked on day 28 (Table 1). The increase in SAP above baseline was
175 significant on day 28 ($p < 0.01$) and 56 ($p < 0.05$), but not anymore on d84. Elevations of DAP
176 and MAP were significant on d28, d56 and d84. SAP, DAP and MAP decreased to baseline
177 values within the first 5 days after hydrocortisone withdrawal, and did not change over the
178 following 11 weeks (Table 1).

179 **Proteinuria**

180 **Urine protein:creatinine ratio**

181 Initially, UPC was below 0.4 in all dogs (0.17; 0.13-0.38). No change in UPC was seen in
182 control dogs throughout the study (Table 2). In the I-HC group, UPC ratios increased
183 significantly ($p < 0.0001$) during hydrocortisone administration to a peak on day 28. UPC ratios
184 on day 28 and day 84 were significantly higher compared to day 1 and 2 (Fig. 1). Compared to

185 the control group UPC ratios in the I-HC group were significantly higher on day 28 and 56. Only
186 two out of six dogs in the hydrocortisone group developed clinically significant proteinuria
187 defined as UPC ratio $> 0.5^{16}$, with maximal values in these two dogs of 1.78 and 1.63. One of
188 these dogs (UPC ratio 1.78) showed pyuria with 8-12 leucocytes, results of urine cultures were
189 negative in both these (and all the other dogs throughout the study), and urine electrophoresis
190 showed a strong band in the molecular weight range of 65-70 kDa corresponding to albumin in
191 both dogs. On d84 the dog with pyuria again showed an increased UPC ratio of 1.63, this time
192 without pyuria, a negative urine culture and still a single band in the molecular weight range of
193 65-70 kDa.

194 In both groups there was a significant, but weak correlation between UPC ratios and SAP ($r =$
195 0.611 , $p = 0.0001$ in control dogs and $r = 0.634$, $p = 0.0005$ in the I-HC group). After
196 hydrocortisone withdrawal UPC gradually decreased, reaching pre-treatment values within one
197 month.

198 Male dogs consistently had a higher UPC ratio than female dogs, except on d28, d56 and d84
199 under hydrocortisone treatment, i.e. at times when urinary protein excretion was affected by
200 hydrocortisone administration (Table 2).

201 **Microalbuminuria and urine albumin:creatinine ratio**

202 Before starting treatment all dogs in the I-HC group were found to be negative for MALB,
203 whereas 2 male dogs in the control group were positive (++) . During treatment 4 dogs in the I-
204 HC group developed variable degrees of MALB (+, ++, or +++) on days 28, 56 and 84, but not
205 consistently. One month after stopping hydrocortisone administration MALB was no longer
206 detectable in these 4 dogs. One dog that was negative for MALB during hydrocortisone
207 administration was positive (++) on day 1 and 5 after hydrocortisone withdrawal. The two

208 dogs in the control group that were positive before the start of treatment remained positive, and
209 all other control dogs remained negative throughout the study.
210 UAC ratios before treatment were 0.017 (0.005 – 0.082) in the control group and 0.015 (0.009 –
211 0.023) in the I-HC group. No change in UAC was seen in control dogs throughout the study
212 (Table 2). UAC ratios gradually increased during hydrocortisone administration (Fig. 2) reaching
213 a maximum on day 84, which showed a 13-fold (2.5 – 73.6) increase. UAC ratios were
214 significantly higher on day 84 compared to baseline and day 1, and on day 56 compared to day 1.
215 After stopping hydrocortisone administration UAC progressively decreased and returned to
216 baseline values within 28 days. UAC ratios in the two control dogs which were positive in the
217 MALB test throughout the study, ranged between 0.044 and 0.098 (median 0.067), and 0.048
218 and 0.113 (median 0.081), respectively.

219 **Correlation between the microalbuminuria test and urine albumin:creatinine ratio**

220 There was a moderate and significant correlation between UAC and the MALB concentrations (r
221 = 0.719, $p < 0.0001$). Overall 105 samples had a negative MALB result, 31 had a + result, four a
222 ++, and four a +++ result. UAC ratios in the different categories of MALB were 0.002-0.131
223 (median 0.012) for negative, 0.044-0.242 (0.079) for low positive, 0.164-0.725 (0.325) for
224 medium positive and 0.242-1.251 (1.023) for strong positive results. At a UAC cut-off of 0.078
225 (mean+2SD in control dogs), sensitivity and specificity of the MALB were 81% and 88%,
226 respectively. At a UAC cut-off of 0.03, sensitivity and specificity of the MALB were 64% and
227 100%, respectively.

228 **Urine protein electrophoresis**

229 In the urine of the dogs two distinct bands were found by SDS-AGE, one in the molecular weight
230 range of 65-70 kDa corresponding to albumin and the other in the range of 25-30 kDa. The

231 patterns in these dogs were characterized by either no band, an isolated band of 65-70 kDa, an
232 isolated band of 25-30 kDa or both bands (Fig. 3A). Each dog of the I-HC group developed a
233 band or showed a pronounced increase in the density of the band in the range of 65-70 kDa.
234 After stopping hydrocortisone administration, the intensity of the albumin band decreased
235 starting on day 5 and patterns were similar to baseline in each dog at d28p. A representative
236 example of an SDS-AGE for one of these dogs is depicted in Fig. 3B.

237 The band in the range of 25-30 kDa was only present in urine samples from male dogs. Each
238 male dog showed this band during the study period, but not on every occasion. The sequence
239 analysis of the tryptic peptides by LC-MSMS resulted in a sequence coverage of 6-16%. The
240 partial sequences AVIRPGEDR (Fig. 4) and SHDLMLLHLEEPAK of canine arginine esterase
241 were sequenced in all tested protein bands with a Mascot significance threshold of 0.05
242 searching against the full Uniprot database. In some of the samples MSMS spectra matching the
243 peptides SFIHPLYK and VMPHLMWIK were found. There was no additional significant
244 Mascot hit for another protein in this band.

245

246 DISCUSSION

247 Hydrocortisone administration consistently increased BP in the dogs in this study. Whereas in
248 humans a significant rise in SBP has been described within as little as 24 hours of commencing
249 cortisol administration^{2,17} the rise in our dogs was not significant on days 1 and 5, but only on
250 day 28. In contrast to dogs with spontaneous HC, where hypertension is a common finding and
251 may be marked¹⁸, no dog in our study developed increased BP to the point of hypertension
252 defined as SAP > 160 mmHg.¹⁹ As a matter of fact, the increase in SAP averaged only 20 mmHg
253 (12-21 mmHg) within 1-2 months of hydrocortisone administration and thereafter even tended to

254 decrease during the treatment period. Recently, categorization of BP has been recommended due
255 to risk of developing subsequent target organ damage. Applying this new classification scheme
256 of hypertension, all dogs stayed in the lowest risk category (BP < 150/95 mmHg).²⁰
257 This discrepancy may have different causes. First, glucocorticoids may not be a relevant
258 contributor to hypertension in spontaneous HC, second, the prevalence of hypertension in
259 spontaneous HC may have been overestimated and third, our model may not closely resemble
260 spontaneous HC. In dogs with spontaneous HC hormones other than cortisol may be responsible
261 for the development of hypertension. Although in HC hypercortisolemia is considered to be of
262 primary pathophysiological importance in dogs, excess production of aldosterone and precursors
263 of the glucocorticoid and mineralocorticoid pathways, i.e. 17a-OH-pregnenolone, 17a-OH-
264 progesterone, 11-deoxycortisol, 21-deoxycortisol and corticosterone have been reported.^{10,21,22}
265 Of these, certainly aldosterone, but also various precursors may play a role in the development of
266 hypertension.^{23,24} BP and the prevalence of hypertension may have been overestimated in clinical
267 studies on HC. It has been shown that BP values strongly depend on acclimation to the
268 measurement procedure^{25,26}, with decreasing values over several days of measurements.
269 Therefore, if BP measurements are obtained on a single day only, false high readings may result
270 and hypertension may be overdiagnosed. A limitation of our model may be that our experimental
271 dogs were only 3.5 years old, whereas spontaneous HC is typically a disease of middle-aged to
272 older dogs. BP has been shown to increase with age²⁷; thus, confounding factors related to age,
273 e.g. atherosclerosis and obesity, may play a role in developing hypertension in spontaneous HC,
274 whereas in our experimental healthy dogs excessive hydrocortisone was the only variable. Also,
275 in spontaneous HC the cortisol excess may be more gradual but present for a much longer time.
276 In this respect it is interesting to note, however, that blood pressure did not continue to increase

277 in the third month of hydrocortisone administration but tended, rather, to decrease, suggesting
278 that there is no linear relationship between duration of HC and increasing blood pressure.
279 Finally, with administration of hydrocortisone twice a day we created only two plasma peaks of
280 cortisol, whereas in spontaneous HC there are multiple peaks throughout the day.²⁸
281 As indicated, not only was the increase in BP mild, but also SAP again started to decrease after
282 d28. An explanation for this finding may be that chronic glucocorticoid treatment results in some
283 metabolic adaptation. Glucocorticoid receptor downregulation in reaction to GC therapy was
284 demonstrated in vitro and in vivo,^{29,30} but the possible mechanism of this receptor
285 downregulation is poorly understood. There is evidence for an enhanced receptor degradation
286 and a modulated GR expression.^{31,32} A recent study showed that in humans with endogenous HC
287 not a GR downregulation but a diminished ligand affinity might partially protect the cells from
288 the high cortisol levels.³³ Another explanation for this finding may be that measurement on day
289 56 and 84 fell into the months of July and August, i.e. the hottest months of the year. High
290 temperatures result in vasodilatation, decreasing peripheral vascular resistance and reduction in
291 BP.³⁴ Also, extracellular volume due to water loss by panting and impaired urine concentrating
292 ability may have been lower in dogs with HC. However, all dogs in the study had free access to
293 water and a cool environment, and hematocrit was not different over time between groups, thus
294 there was no reason to suspect hypovolemia.
295 In all our experimental dogs BP returned to basal values shortly after cessation of hydrocortisone
296 administration. In contrast, in dogs and humans with spontaneous HC hypertension often persists
297 despite appropriate treatment and improvement of other clinical signs.^{4,35,36} This again indicates
298 that hypertension in spontaneous HC is not just caused by the cortisol excess per se. The
299 persistence of hypertension in spontaneous HC could be the result of irreversible peripheral

300 vascular remodeling followed by increased wall stiffness and increased vascular resistance. This
301 hypothesis is supported by the finding that patients with a history of hypertension of less than 5
302 years were more likely to become normotensive after adrenalectomy.³⁷ However, the factors that
303 influence persistence of hypertension have not been studied in either humans or dogs.

304 In addition to an elevated BP we observed increased urinary protein excretion in hydrocortisone
305 treated dogs. The increases in UPC ratios in most dogs were mild and smaller compared to dogs
306 with spontaneous HC⁴ or dogs receiving prednisone for 42 days.⁸ The increase in proteinuria is
307 thought to be primarily of glomerular origin, as there was only albuminuria as determined by
308 SDS-AGE. Nevertheless, proteins other than albumin that may have been in the urine of our
309 dogs were lost because the increase in UAC does not account for the increase in UPC, i.e. UPC
310 was higher than UAC; thus, SDS-AGE was not sensitive enough to detect small amounts of
311 multiple various proteins.

312 Possible causes of increased glomerular proteinuria include increased intraglomerular pressure
313 and damage to the glomerular basement membrane. Elevated BP and a significant albeit weak
314 positive correlation between SAP and UPC in our dogs supports hypertension as being one
315 causative mechanism of proteinuria. Even though systemic hypertension does not directly
316 translate into increased intraglomerular pressure, glucocorticoids have also been shown to
317 increase renal plasma flow and glomerular filtration rate in dogs.^{38,39,40} Thus, glucocorticoids
318 may mediate proteinuria by hemodynamic alterations resulting in an increase in glomerular
319 pressure. Altered renal histomorphology was found in dogs treated with prednisone for 42 days.⁸
320 The most consistent finding was mild to moderate hypercellular glomerular tufts, suggestive of
321 mesangial proliferation. Other findings were glomerular adhesions and moderately thickened
322 Bowman's capsules. Electron microscopy was characterized by occasional mild segmental

323 thickening of basement membranes, fusion of visceral cell foot processes and glomerular
324 adhesions. Lack of pre-treatment renal histologic examination, lack of a control group and lack
325 of follow-up histologic examination after discontinuation of glucocorticoids makes it difficult to
326 say whether these changes are a result of glucocorticoid administration and whether histologic
327 changes are reversible. As in our study no renal histologic examinations were performed, our
328 results cannot answer the question of whether the quickly reversible proteinuria was due to either
329 functional or morphological changes, or both. In spontaneous HC, proteinuria may or may not
330 significantly decrease when the disease is well controlled.^{4,7,41} Presumably, elevated
331 intraglomerular pressure in HC will lead to reversible or irreversible morphologic changes,
332 depending on degree and chronicity.

333 The 17% prevalence (2/12) of MALB in the healthy dogs in this study before any treatment is in
334 accordance with other studies reporting a prevalence in healthy dogs ranging between 15 and
335 19%.^{42,1} It is important to note that there may be significant day-to-day variations of up to 30 -
336 80% in daily urinary albumin excretion in man.⁴³ This variation is dependent on posture,
337 exercise, and dietary factors, such as protein intake.^{44,45} Data from human patients indicate that
338 MALB is associated with an increased cardiovascular risk in hypertensive patients^{46,47} and the
339 nondiabetic population.^{48,49} In our two healthy dogs follow-up assays were positive throughout
340 the study, and they are therefore thought to be microalbuminuric, however, the significance of
341 this finding is unknown. The most important consideration when assessing MALB should
342 probably be the context of where this result is obtained, i.e. is it any patient or a patient at risk of
343 end organ damage. In this respect, the authors are unaware of any data that associate MALB with
344 increased morbidity or mortality in dogs.

345 MALB can be quantitated by different means. Enzyme-linked immunoassay analysis,
346 nephelometry and immunoturbidimetry are complicated, time consuming and expensive
347 laboratory techniques, unsuitable for routine use. Recently, the ERD HealthScreen[®] test, a simple
348 and fast test for the semiquantitative detection of MALB has been commercialized. In the present
349 study, to verify the accuracy of MALB observed in healthy dogs and in dogs with iatrogenic HC
350 as measured by this simple screening test we performed immunoturbidimetry as a quantitative
351 reference method. In human medicine, an UAC ratio > 0.03 is considered abnormal and
352 indicative of pathological MALB.¹³ Unfortunately few data exist on the normal range of UAC in
353 healthy dogs, but in a previous study mean UAC ratio in 10 healthy dogs was 0.04 ± 0.08^m . The
354 two healthy dogs with MALB were several times above the level of 0.078 and thus correctly
355 identified with the screening test. Based on our findings the ERD HealthScreen[®] test appears to
356 be an efficient and fast method for the semiquantitative detection of MALB in dogs with good
357 correlation to a quantitative laboratory method.

358 Higher UPC in male dogs when urinary protein excretion is not affected by hydrocortisone
359 administration is explained by the distinct protein band in the molecular weight range of 25 to 30
360 kDa, identified as arginine esterase. This enzyme, the major secretory protein of the prostatic
361 gland, enters the bladder by reflux from the proximal urethra.^{50,51} An isolated band in the same
362 molecular weight range has recently been described as free light chains and linked to male *and*
363 female dogs exposed to or infected with certain infectious agents; i.e. *Leishmania infantum*,
364 *Ehrlichia canis* and *Babesia canis*.⁵² On the basis of our results, this protein band is a
365 physiological finding in urine samples of intact male dogs, i.e. arginine esterase, and does not
366 necessarily represent free light chains in every case.

367 In conclusion, our study showed that long-term hydrocortisone administration induces mild
368 elevation of BP and mild proteinuria mainly characterized by albuminuria, suggesting proteinuria
369 of glomerular origin. Both hypertension and proteinuria were reversible and completely restored
370 within one month after cessation hydrocortisone administration. The higher UPC ratio in intact
371 male dogs compared to female dogs can be explained by the physiological presence of arginine
372 esterase.

- 373 ^a Hydrocortisone tablets, Hotz Pharmacy, Kusnacht, Switzerland
- 374 ^b Synacthen[®], Novartis Pharma Schweiz AG, Bern, Switzerland
- 375 ^c Schellenberg S, Reusch CE, Glaus TM. Effect of adaptation on indirect blood pressure
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- 379 ^e Canine E.R.D.-HealthScreen[®] Test, Heska Corp, Fort Collins, CO
- 380 ^f Microalbumin OSR6167 Olympus system reagent, Olympus Diagnostica GmbH, Clare, Ireland
- 381 ^g Hydragel 5 Proteinurie, semiautomated Hydrasys, Sebia, Fulda, Germany
- 382 ^h Promega
- 383 ⁱ Thermo Finnigan
- 384 ^j SPSS 11.0 for Windows, SPSS Inc., Chicago, IL,
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522 LEGENDS

523 **Fig 1.** Change in urine protein:creatinine ratios (UPC) in 12 dogs before, during and after
524 administration of placebo (n = 6) or hydrocortisone (n = 6). An asterisk (*) indicates significant
525 difference between the different time points. The grey background indicates the hydrocortisone
526 or placebo treatment period.

527
528 **Fig 2.** Change in urine albumin:creatinine ratios (UAC) in 12 dogs before, during and after
529 administration of placebo (n = 6) or hydrocortisone (n = 6). An asterisk (*) indicates significant
530 difference between the different time points. The grey background indicates the hydrocortisone
531 or placebo treatment period.

532
533 **Fig 3.** Analysis of urine protein patterns by agarose gel electrophoresis followed by staining with
534 acid violet. The different protein patterns found in urine samples obtained from dogs in the
535 control group (A). The change in urine protein pattern in a hydrocortisone treated female dog at
536 the different time points (B). M, marker; m, male dog; f, female dog; Lxy, Lane xy

537
538 **Fig 4.** Representative MSMS spectrum of the tryptic peptide AVIRPGEDR of canine ariginine
539 esterase (ESTA_CANFA) identified by Mascot. The major fragments generated are labeled and
540 the y- and b- fragments detected are indicated in the sequence.

Table 1. Median values and ranges of systolic (SAP), diastolic (DAP) and mean arterial blood pressure (MAP) in control and hydrocortisone treated dogs at the different time points.

	SAP (mmHg)		DAP (mmHg)		MAP (mmHg)	
	Controls Median (Range)	I-HAC Median (Range)	Controls Median (Range)	I-HAC Median (Range)	Controls Median (Range)	I-HAC Median (Range)
d0	128 (122 - 142)	123 (114 - 136)	63 (55 - 83)	69 (53 - 78)	90 (78 - 104)	90 (81 - 101)
d1	128 (123 - 135)	130 (118 - 136)	68 (63 - 75)	70 (66 - 78)	89 (82 - 95)	91 (84 - 99)
d5	126 (117 - 148)	133 (122 - 144)	66 (56 - 77)	75 (64 - 81)	89 (80 - 98)	94 (82 - 103)
d28	129 (116 - 149)	143 (128 - 148) ^a	70 (65 - 83)	81 (77 - 90) ^a	94 (83 - 107)	102 (95 - 109) ^a
d56	130 (122 - 145)	137 (134 - 139) ^a	72 (63 - 86)	82 (74 - 82) ^a	96 (84 - 105)	103 (96 - 105) ^a
d84	127 (119 - 144)	131 (129 - 141)	66 (59 - 81)	80 (71 - 81) ^a	89 (80 - 106)	98 (94 - 104) ^a
d1p	129 (118 - 141)	130 (124 - 144)	66 (54 - 75)	78 (65 - 84)	91 (80 - 103)	98 (89 - 103)
d5p	133 (117 - 152)	124 (106 - 136)	73 (53 - 86)	70 (55 - 76)	94 (77 - 115)	88 (77 - 98)
d28p	131 (123 - 145)	124 (117 - 128)	66 (60 - 86)	69 (61 - 81)	91 (86 - 105)	87 (84 - 97)
d56p	126 (118 - 140)	124 (116 - 130)	70 (59 - 81)	70 (61 - 82)	91 (85 - 104)	87 (79 - 101)
d84p	130 (121 - 143)	127 (117 - 133)	67 (60 - 79)	68 (62 - 77)	89 (79 - 107)	91 (85 - 95)

dx, day x of hydrocortisone treatment, dxp, day x after hydrocortisone treatment

^a p < 0.05 versus d0

Table 2. Median values, ranges and P-values (control vs. I-HC dogs) of urine protein:creatinine (UPC) and urine albumin:creatinine (UAC) ratios in control dogs and dogs of the I-HC group at the different time points.

	UPC		P value	UAC		P value
	control dogs Median (Range)	I-HC dogs Median (Range)		control dogs Median (Range)	I-HC dogs Median (Range)	
d0	0.21 (0.13 - 0.38)	0.17 (0.15 - 0.28)	0.937	0.017 (0.005 - 0.082)	0.015 (0.009 - 0.023)	0.937
d1	0.15 (0.12 - 0.32)	0.12 (0.09 - 0.22)	0.240	0.014 (0.006 - 0.060)	0.013 (0.010 - 0.028)	0.818
d5	0.18 (0.10 - 0.23)	0.15 (0.08 - 0.28)	0.937	0.013 (0.004 - 0.048)	0.016 (0.005 - 0.043)	1.000
d28	0.19 (0.13 - 0.29)	0.38 (0.18 - 1.78) ^a	0.026	0.024 (0.004 - 0.067)	0.045 (0.012 - 0.795)	0.240
d56	0.19 (0.11 - 0.30)	0.30 (0.20 - 0.51)	0.041	0.016 (0.005 - 0.079)	0.083 (0.021 - 0.339) ^b	0.015
d84	0.22 (0.10 - 0.36)	0.33 (0.21 - 1.63) ^a	0.065	0.020 (0.003 - 0.103)	0.133 (0.034 - 1.251) ^{b,c}	0.026
d1p	0.24 (0.09 - 0.34)	0.34 (0.20 - 1.03)	0.180	0.023 (0.003 - 0.070)	0.101 (0.011 - 0.725)	0.041
d5p	0.24 (0.12 - 0.30)	0.24 (0.19 - 0.67)	0.699	0.035 (0.002 - 0.078)	0.037 (0.016 - 0.311)	0.589
d28p	0.20 (0.08 - 0.36)	0.17 (0.11 - 0.31)	0.937	0.020 (0.002 - 0.093)	0.010 (0.005 - 0.032)	1.000
d56p	0.18 (0.09 - 0.36)	0.18 (0.14 - 0.22)	0.937	0.018 (0.004 - 0.113)	0.013 (0.003 - 0.025)	0.699
d84p	0.25 (0.10 - 0.35)	0.20 (0.13 - 0.30)	0.699	0.027 (0.007 - 0.104)	0.014 (0.005 - 0.037)	0.485

dx, day x of hydrocortisone treatment, dxp, day x after hydrocortisone treatment

^a p < 0.05 versus d1 and d5, ^b p < 0.05 versus d1, ^c p < 0.05 versus d0

Table 3. Median values, ranges and P-values (male vs female dogs) of urine protein:creatinine ratios (UPC) in all male and female dogs at the different time points.

	UPC		P value
	male dogs Median (Range)	female dogs Median (Range)	
d0	0.28 (0.18 - 0.38)	0.15 (0.13 - 0.16)	0.002
d1	0.19 (0.11 - 0.32)	0.13 (0.09 - 0.15)	0.041
d5	0.22 (0.16 - 0.28)	0.12 (0.08 - 0.14)	0.002
d28	0.30 (0.23 - 1.78)	0.17 (0.13 - 1.63)	0.065
d56	0.28 (0.20 - 0.49)	0.20 (0.11 - 0.51)	0.394
d84	0.33 (0.21 - 1.63)	0.24 (0.10 - 0.34)	0.180
d1p	0.34 (0.20 - 1.03)	0.20 (0.09 - 0.39)	0.065
d5p	0.29 (0.19 - 0.67)	0.19 (0.12 - 0.28)	0.026
d28p	0.30 (0.12 - 0.36)	0.11 (0.08 - 0.17)	0.009
d56p	0.22 (0.16 - 0.36)	0.14 (0.09 - 0.19)	0.004
d84p	0.29 (0.21 - 0.35)	0.14 (0.10 - 0.21)	0.002

dx, day x of hydrocortisone treatment, dxp, day x after hydrocortisone treatment

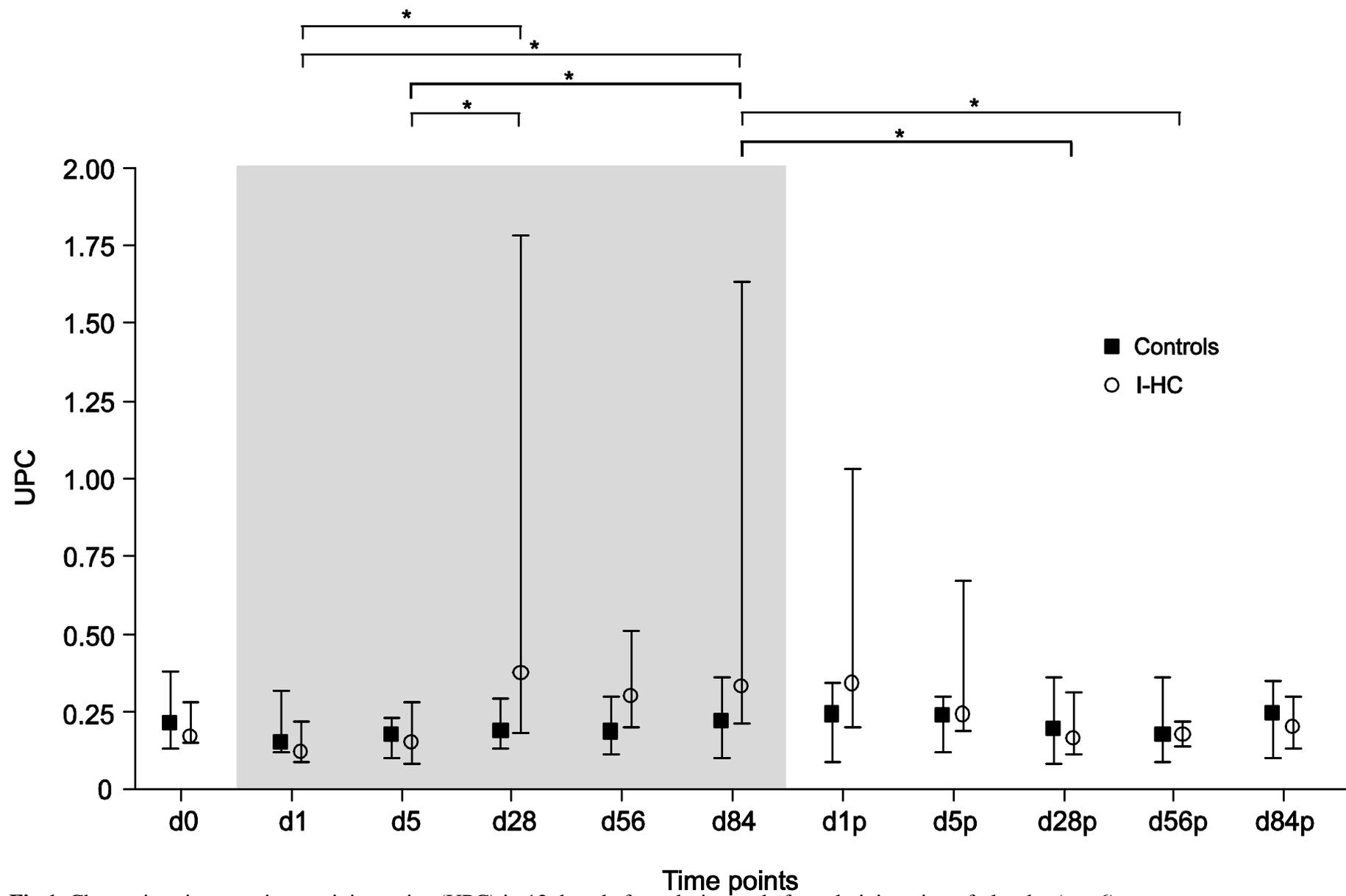


Fig 1. Change in urine protein:creatinine ratios (UPC) in 12 dogs before, during and after administration of placebo (n = 6) or hydrocortisone (n = 6). An asterisk (*) indicates significant difference between the different time points. The grey background indicates the hydrocortisone or placebo treatment period.

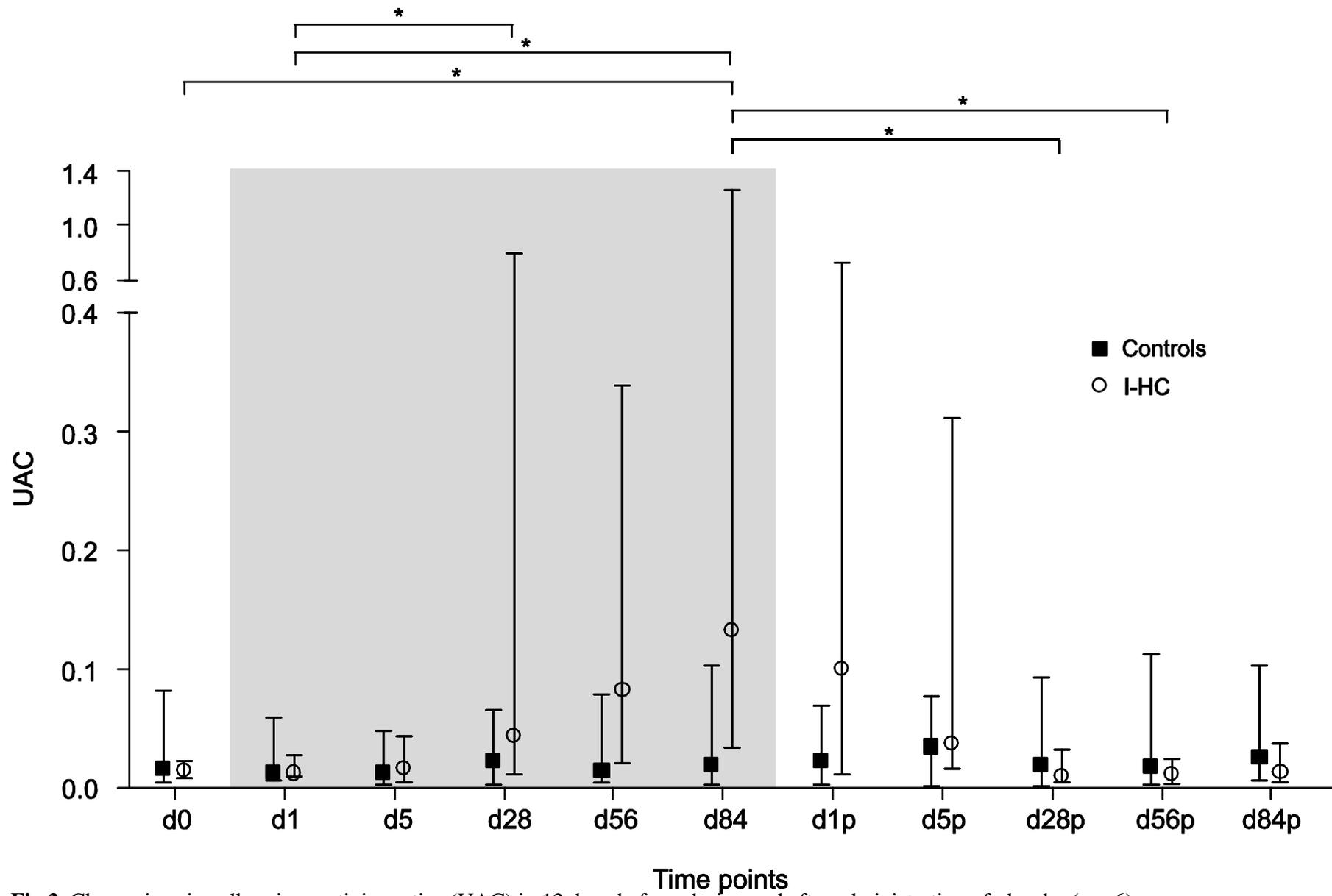


Fig 2. Change in urine albumin:creatinine ratios (UAC) in 12 dogs before, during and after administration of placebo (n = 6) or hydrocortisone (n = 6). An asterisk (*) indicates significant difference between the different time points. The grey background indicates the hydrocortisone or placebo treatment period.

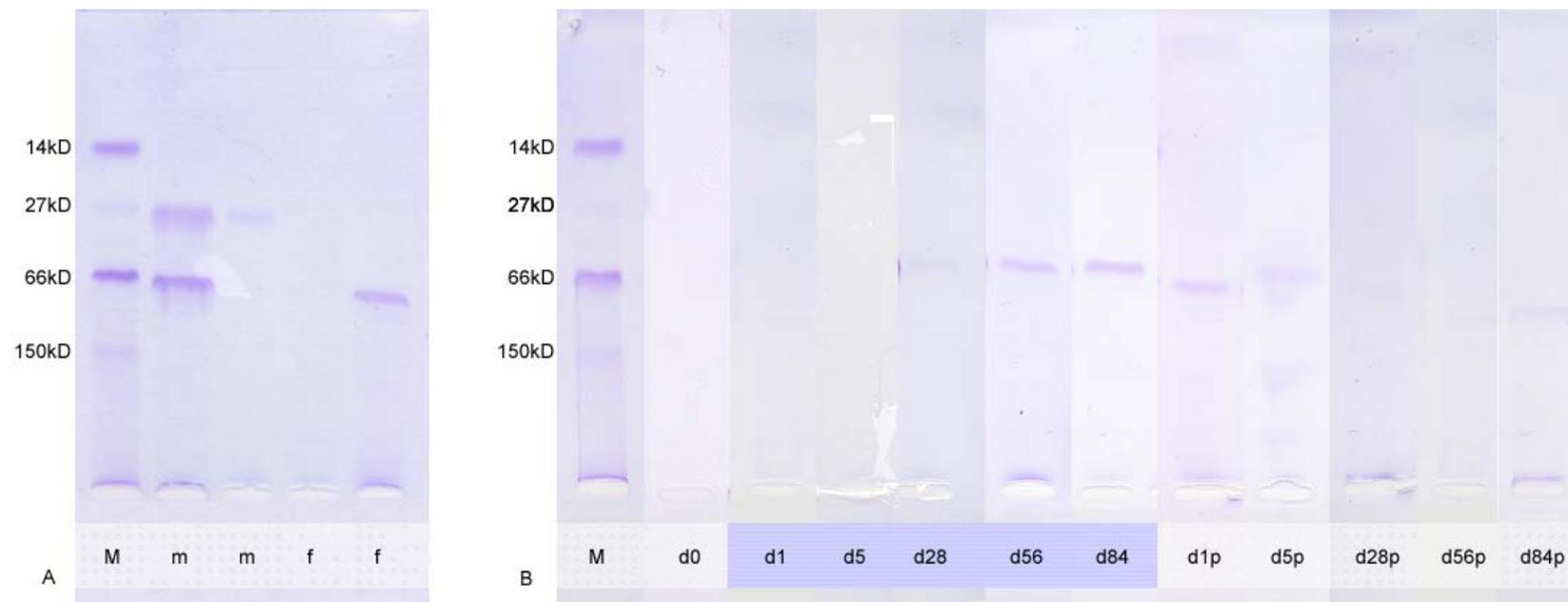


Fig 3. Analysis of urine protein patterns by agarose gel electrophoresis followed by staining with acid violet. The different protein patterns found in urine samples obtained from dogs in the control group (A). The change in urine protein pattern in a hydrocortisone treated female dog at the different time points (B). M, marker; m, male dog; f, female dog.

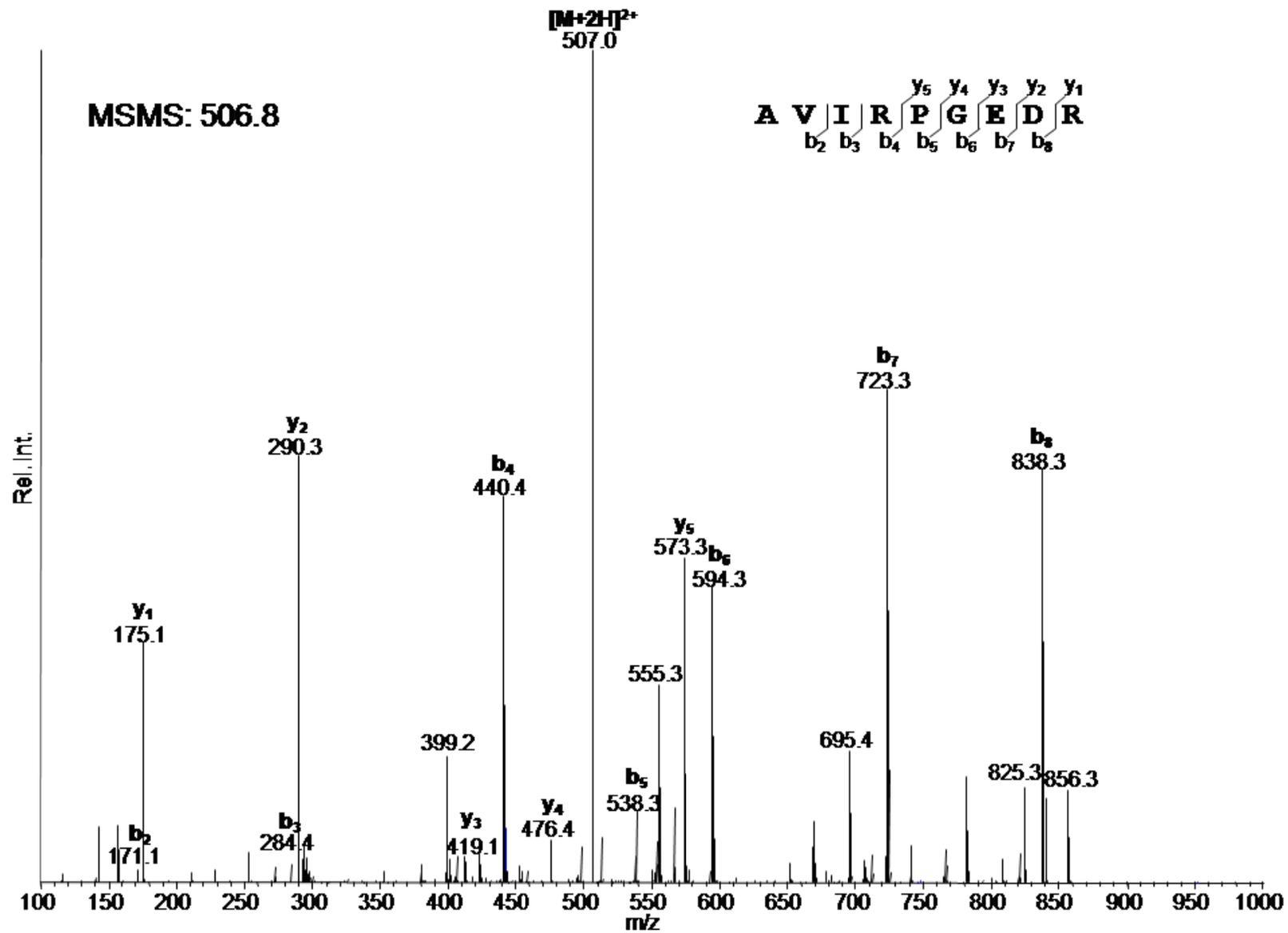


Fig 4. Representative MSMS spectrum of the tryptic peptide AVIRPGEDR of canine arginine esterase (ESTA_CANFA) identified by Mascot. The major fragments generated are labeled and the y- and b- fragments detected are indicated in the sequence.