







FACULTY OF VETERINARY MEDICINE

Department of Small Animal Medicine and Clinical Biology

# **Renal function in dogs with Cushing's syndrome**

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*“In order to keep a true perspective of one's importance,  
everyone should have a dog that will worship him  
and a cat that will ignore him.”*

- Dereke Bruce

Voor Meter en Bompa,  
en voor Oma en Peter



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## LIST OF ABBREVIATIONS

AAP	alanine aminopeptidase	ELISA	enzyme linked immunosorbent assay
ACTH	adrenocorticotropic hormone	GC	glucocorticoid
AH	adenohypophysis	GFR	glomerular filtration rate
AL	anterior lobe	GGT	$\gamma$ -glutamyl transferase
ALB	albumin	HC	hydrocortisone
ALT	alanine aminotransferase	HMW	high molecular weight
AP	alkaline phosphatase	HPA axis	hypothalamus-pituitary-adrenal gland axis
AQP	aquaporin	IgA	immunoglobulin A
ATH	adrenal tumor hypercortisolism	IgG	immunoglobulin G
AUC	area under the curve	IMW	intermediate molecular weight
AVP	arginine vasopressin	IRIS	International Renal Interest Society
BDL	below detection limit	LDDST	low dose dexamethasone suppression test
Ca <sup>2+</sup>	calcium	LDH	lactate dehydrogenase
CKD	chronic kidney disease	LMW	low molecular weight
Cl <sub>creat</sub>	plasma clearance of exogenous creatinine	LOD	limit of detection
Clearance	Cl	MP	methylprednisolone
Cl <sub>endo</sub>	plasma clearance of endo-iohexol	mRNA	messenger ribonucleic acid
Cl <sub>exo</sub>	plasma clearance of exo-iohexol	NAG	N-acetyl- $\beta$ -D-glucosaminidase
CO	cardiac output	NH	neurohypophysis
C <sub>p</sub>	marker concentration in plasma in mg/mL	PDH	pituitary-dependent hypercortisolism
CRH	corticotropin releasing hormone	PEC-ICT	plasma exogenous creatinine-iohexol clearance test
CRP	C-reactive protein	PL	posterior lobe
CS	Cushing's syndrome	RBF	renal blood flow
C <sub>u</sub>	marker concentration in urine in mg/mL	RBP	retinol-binding protein
CV	coefficient of variation	RVR	renal vascular resistance
D	dose	SBP	systolic blood pressure
DEX	dexamethasone	screat	serum creatinine
DNA	deoxyribonucleic acid		

SD	standard deviation
T4	total thyroxine
TPR	total peripheral resistance
u	urinary
U	urine flow in mL/min
uALB/c	urinary albumin-to-creatinine ratio
UCCR	urinary corticoid-to-creatinine ratio
uCRP/c	urinary C-reactive protein-to-creatinine ratio
uIgG/c	urinary immunoglobulin G-to-creatinine ratio
uNAG/c	urinary N-acetyl- $\beta$ -D-glucosaminidase-to-creatinine ratio
UPC	urinary protein-to-creatinine ratio
uRBP/c	urinary retinol-binding protein-to-creatinine ratio
USG	urine specific gravity
XLHN	X-linked hereditary nephropathy
11- $\beta$ -HSD	11- $\beta$ -hydroxysteroid dehydrogenase
<sup>51</sup> Cr-EDTA	<sup>51</sup> Cr-ethylenediaminetetraacetic acid
<sup>99m</sup> Tc-DTPA	<sup>99m</sup> Tc-diethylenetriaminepentaacetic acid





## **CHAPTER I:**

### **GENERAL INTRODUCTION**

**CHAPTER I: *General introduction***

## **CHAPTER I: *General introduction***

In the first part of Chapter I, the focus is on Cushing's syndrome, followed by a second section explaining the link between Cushing's syndrome and the kidney, and ending with an overview of renal function evaluation and a conclusion.

First, physiology of the hypothalamus-pituitary-adrenal gland axis in the dog is briefly explained, to provide the reader with adequate background information to understand the pathophysiology of Cushing's syndrome. Secondly, the clinical presentation, diagnosis and treatment of canine Cushing's syndrome is briefly discussed. Next, the interaction between glucocorticoids and renal function is described, with emphasis on vascular and hemodynamic effects, glomerular filtration rate (GFR), proteinuria and tubular function. In the last section, evaluation of kidney function with routine renal markers as well as GFR and urinary biomarkers are discussed.

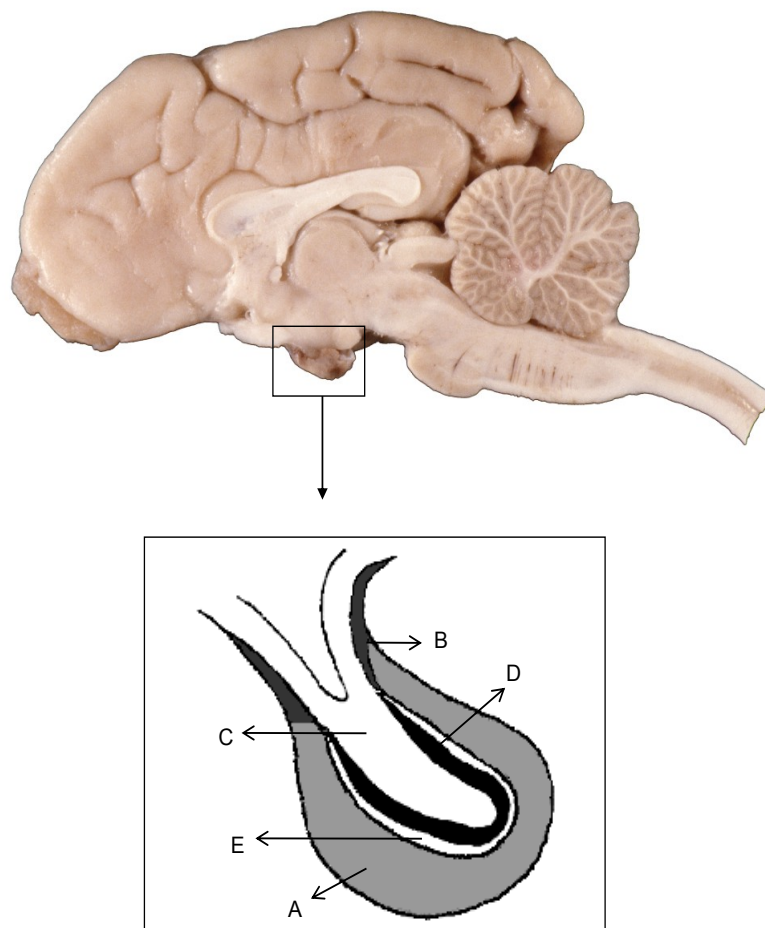
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### 1.1 The hypothalamus-pituitary-adrenal gland axis (HPA-axis)

The canine *pituitary gland* (*hypophysis cerebri*) is connected to the brain in the ventral midline of the diencephalon. As illustrated in Figure 1, it consists of two parts, the *adenohypophysis* (AH) and *neurohypophysis* (NH). The major part of the NH is the *pars distalis NH* (also called *posterior lobe*) of the pituitary gland, which is in close contact with the inner part of the AH, also called *pars intermedia*. The major part of the AH, the *pars distalis AH* or *anterior lobe* (AL) is separated from the *pars intermedia* by the hypophyseal cleft and wraps around the NH like a cuff (*pars tuberalis*).<sup>1</sup>

#### Figure 1. Sagittal section of the canine brain with detail of the pituitary gland.

A: *pars distalis adenophypophysis* (AH), B: *pars tuberalis* AH, C: *pars distalis neurohypophysis* (NH), D: *pars intermedia* AH, E: hypophyseal cleft.





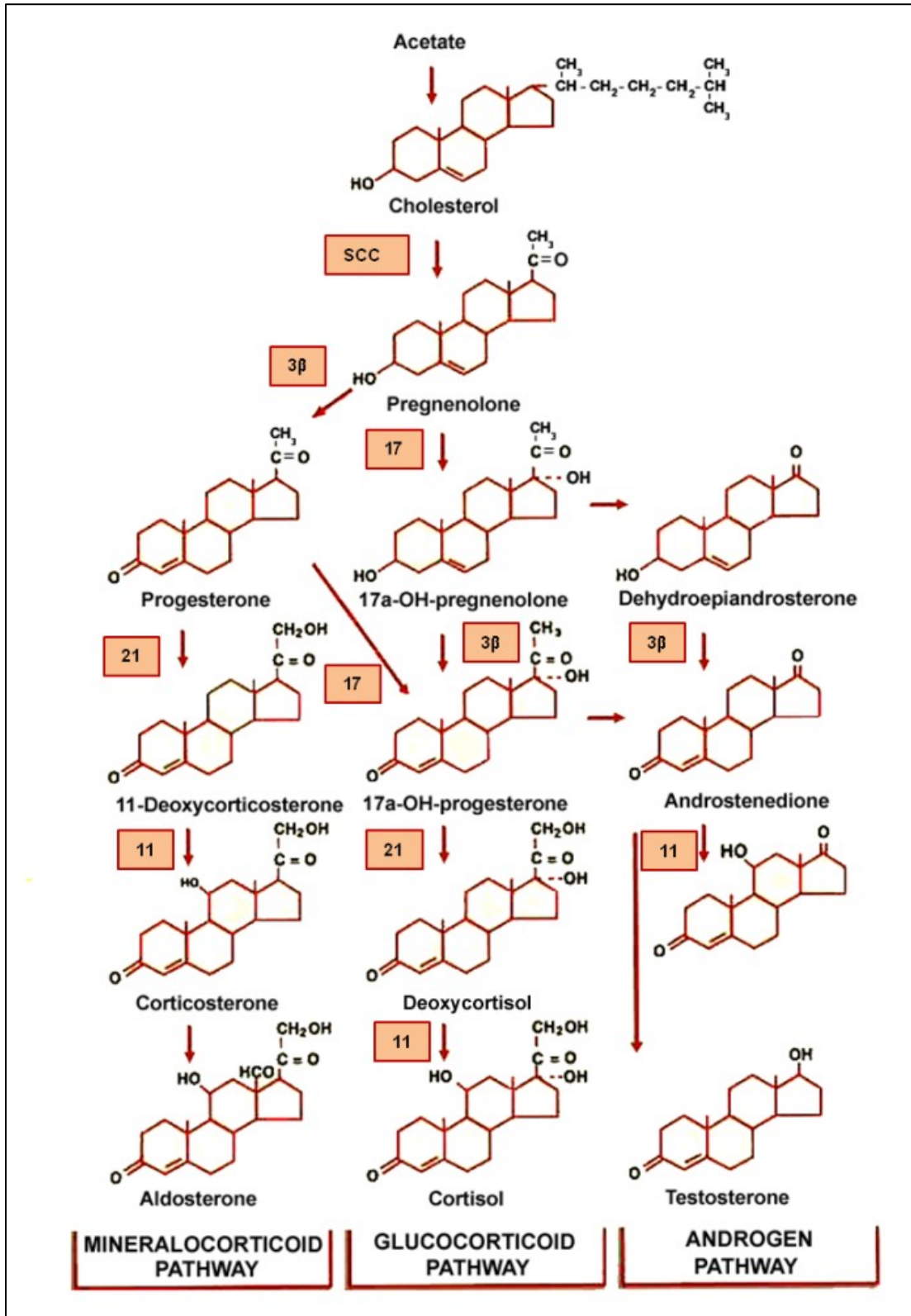
Connected by a portal circulation, neurosecretory pathways and direct neural control, the pituitary gland acts as a functional unit together with the hypothalamus. Release hormones are secreted from hypothalamic axon terminals into portal blood veins, providing access to the five main endocrine cell types of the AL: somatotropic, gonadotropic, lactotropic, thyrotropic and corticotropic cells. This will lead to secretion of their respective hormones: growth hormone, follicle-stimulating hormone and luteinizing hormone, prolactin, thyroid stimulating hormone and adrenocorticotrophic hormone (ACTH), although recently multi-responsive cells with receptors for different release hormones have also been found.<sup>2</sup> These hormones target endocrine glands such as gonads, thyroid and adrenal glands.

The adrenal glands are paired organs located craniomedially near the kidneys and consist of two functionally distinct areas: the inner part or *medulla*, producing catecholamines such as epinephrine and norepinephrine and the outer part or *cortex*, secreting steroid hormones such as cortisol, aldosterone and androgens. Histologically, three zones can be distinguished in the cortex: the *zona reticularis* and *zona fasciculata* which produce cortisol and androgens and the *zona glomerulosa*, in which aldosterone is synthesized. Only cells of the *zona fasciculata* and *reticularis* contain 17- $\alpha$ -hydroxylase, the enzyme necessary for synthesis of cortisol- and androgen-precursors 17- $\alpha$ -hydroxypregnenolone and 17- $\alpha$ -hydroxyprogesterone.<sup>3</sup> Steroid hormones are synthesized by enzymatic action on cholesterol as shown in Figure 2. Conversion of cholesterol to pregnenolone in the mitochondria is the rate-limiting step and this is regulated primarily by ACTH through activation of adenylyl cyclase, increase of cyclic adenosine monophosphatase and activation of phosphoprotein kinases.<sup>4</sup>

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**Figure 2. Pathways of adrenocortical steroid biosynthesis.**

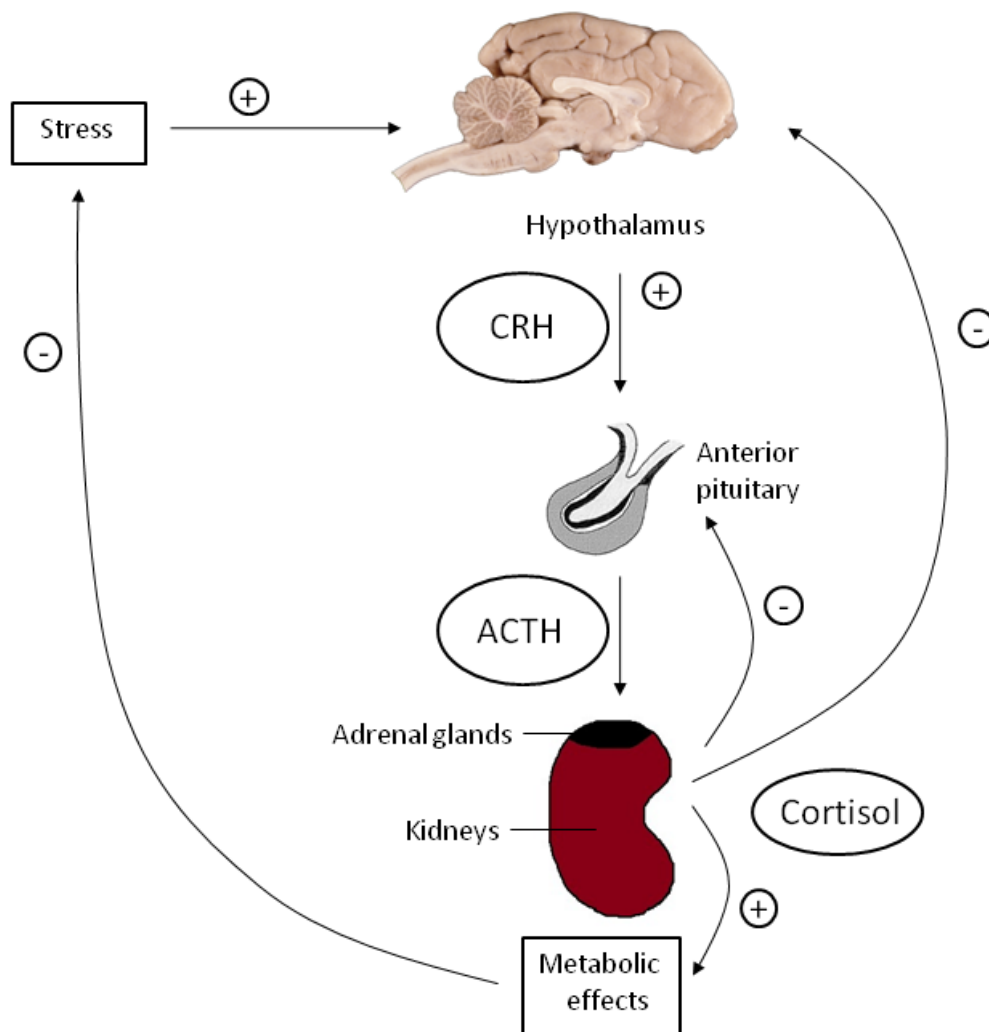
SCC: cholesterol side chain cleavage, 3 $\beta$ : 3 $\beta$ -hydroxysteroid dehydrogenase, 11: 11 $\beta$ -hydroxylase, 17: 17 $\alpha$ -hydroxylase, 21: 21-hydroxylase. Adapted from Rijnberk A. and Kooistra H.S., 2010. *Clinical Endocrinology of Dogs and Cats*, p. 95.



Cortisol secretion is mainly controlled by the HPA axis as depicted in Figure 3. Several physical, emotional and chemical stress stimuli stimulate secretion of corticotropin release hormone (CRH) by the hypothalamus, leading to secretion of ACTH by corticotropic cells of the pituitary AL into the systemic circulation. This is followed by synthesis and secretion of cortisol by the adrenal gland. Cortisol will in its turn cause a long-loop negative feedback signal to the AL and the hypothalamus, thus decreasing its own secretion. Adrenocorticotrophic hormone also limits its own production via a short-loop feedback mechanism to the hypothalamus.<sup>4</sup>

**Figure 3. Hypothalamus-pituitary-adrenal gland axis.**

+, stimulation signal; -, negative feedback signal; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotrophic hormone.



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Cortisol and synthetic glucocorticoids exert their effects in many tissues by interaction with the glucocorticoid receptor, but also through mineralocorticoid receptors and their tissue-specific activity is mediated by the microsomal enzyme 11- $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD).<sup>5</sup> There are two distinct isotypes of 11 $\beta$ -HSD: 11 $\beta$ -HSD type 1 (11 $\beta$ -HSD1) predominantly has reductase activity converting cortisone to biologically active cortisol, whereas 11 $\beta$ -HSD type 2 (11 $\beta$ -HSD2) oxidises cortisol to inactive cortisone. Although 11 $\beta$ -HSD1 is found in most tissues, 11 $\beta$ -HSD2 is preferentially located in mineralocorticoid target organs, such as the kidney.<sup>6,7</sup>

After lipophilic glucocorticoids permeate the cell membrane, they bind to a cytosolic receptor protein and the glucocorticoid-receptor complex then enters the nucleus to interact with specific sites of the deoxyribonucleic acid (DNA). This leads to changes in messenger ribonucleic acid (mRNA) transcription and subsequent synthesis of specific proteins, eliciting the glucocorticoid response.<sup>3,8</sup> Gene expression inhibition is a key glucocorticoid action, responsible for anti-inflammatory effects and catabolic effects on muscle, bone and connective tissue. In the liver, however, glucocorticoids activate mRNA transcription and synthesis of important enzymes for gluconeogenesis.<sup>3,8</sup> Through metabolic effects and other mechanisms, glucocorticoids affect almost all tissues. Therefore, cortisol excess, either endogenous or by administration of exogenous glucocorticoids, leads to multiple clinical signs and laboratory abnormalities that are referred to as “Cushing’s syndrome”, which will be discussed in the following section.

## 1.2 Canine Cushing's syndrome

### 1.2.1 Etiology and clinical presentation

The clinical and biochemical abnormalities resulting from chronic overexposure to glucocorticoids are referred to as hypercortisolism or *Cushing's syndrome*, after Dr. Harvey Cushing, who first described this disease in people in the 1930s.<sup>9</sup> In 80 to 85% of canine cases, chronic glucocorticoid excess is caused by increased ACTH secretion by a pituitary tumor, this is also referred to as *Cushing's disease* or pituitary-dependent hypercortisolism (PDH). Secondly, adrenocortical tumors or ACTH-independent hypercortisolism account for about 15 to 20% of cases of spontaneous hypercortisolism in dogs.<sup>3</sup> Most adrenocortical tumors are unilateral solitary lesions and can be carcinoma's or adenoma's, with bilateral tumors occurring in about 10% of cases.<sup>10-12</sup> In a minority of human patients, a third form of Cushing's syndrome results from ectopic ACTH secretion caused by carcinoid tumors or small-cell lung carcinomas.<sup>13</sup> In dogs, only two case reports describe Cushing's syndrome as a result of ectopic ACTH secretion caused by carcinoid tumors.<sup>14,15</sup> Rarely, aberrant expression of ectopic or overactive eutopic receptors in adrenal glands cause adrenal hypercortisolism in people and there is one case-report describing this fourth variety of Cushing's syndrome in a dog.<sup>16,17</sup>

Cushing's syndrome is one of the most common endocrine disorders in middle aged to older dogs, with an estimated incidence of 1 to 2 cases/1000 dogs/year.<sup>18</sup> Cushing's disease has been diagnosed in virtually all dog breeds, with a possible predilection for small breed dogs such as miniature poodles and dachshunds. Poodles, German shepherd dogs, labrador retrievers and terriers appear among the most frequently affected breeds with adrenal tumor hypercortisolism (ATH).<sup>19</sup> Typical clinical signs of both PDH and ATH are polyuria and polydipsia, polyphagia, central obesity or "pot-belly", muscle wasting and skin changes, including atrophy, alopecia and comedones. A dog with typical "Cushingoid" features is presented in Figure 4. As previously stated, glucocorticoids act on many tissues and the clinical manifestations resulting from glucocorticoid excess are shown in Table 1.

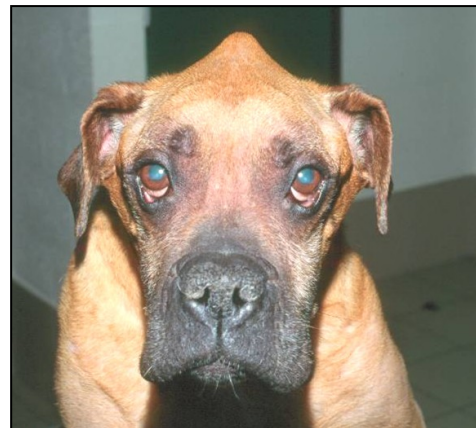
**CHAPTER I: General introduction**

**Table 1. Clinical manifestations of glucocorticoid excess in dogs.**

Pp, polyphagia; pu/pd, polyuria and polydipsia; AP, alkaline phosphatase; ALT, alanine aminotransferase; T4, thyroxine. Adapted from Rijnberk A. and Kooistra H.S., 2010. Clinical Endocrinology of Dogs and Cats, p. 114.

System	Common	Less common
Urinary	pu/pd, proteinuria	urinary tract infection, glucosuria
Skin and hair	thin coat, alopecia, skin atrophy, comedones	hyperpigmentation, calcinosis cutis
Respiratory/ Cardiovascular	panting at rest, hypertension	congestive heart failure, pulmonary thrombo-embolism
Metabolic	pp, weight gain hepatomegaly, pot-belly	weight loss (muscle wasting), intolerance to hot environment
Neuromuscular	lethargy, muscle weakness, muscular atrophy	myotonia
Reproductive	absence of estrus	testicular atrophy
Hematology and biochemistry	neutrophilia, eosinopenia, lymphopenia, increased AP and ALT, low T4, hypercholesterolemia, hyperlipidemia	increased hematocrit value, hyperglycemia, hypernatremia, hypokalemia

**Figure 4. Cushingoid features in a Boxer dog.** Note the "pot-belly", skin atrophy and alopecia on the abdomen and limbs, lordosis and severe atrophy of the masticatory muscles. Pictures courtesy prof. S. Daminet.



### **1.2.2 Diagnosis**

Diagnosis of Cushing's syndrome is based on history and clinical signs, physical examination, endocrine function tests and medical imaging.

Frequent hematology and biochemical abnormalities include a stress leukogram (neutrophilia, monocytosis, lymphopenia, eosinopenia), thrombocytosis, increased alkaline phosphatase, increased cholesterol and triglycerides. Urinalysis often indicates dilute urine (urinary specific gravity, USG < 1.020) and sometimes signs of a urinary tract infection.<sup>8</sup>

When a patient is suspected of Cushing's syndrome, the diagnosis must be confirmed using endocrine screening tests. An easy and minimal invasive test is measurement of urinary corticoid-to-creatinine ratios (UCCR) in two consecutive morning urine samples. To avoid influence of stress, urine samples must be collected by the owner in the dog's home environment.<sup>20</sup> Other "stress-factors" like concomitant disease can also cause falsely elevated UCCRs, which decreases the specificity of the test.<sup>21,22</sup> With a negative predictive value of 0.98, the UCCR is a very sensitive test, and therefore a good screening method.<sup>23</sup> A second screening test is the low-dose dexamethasone suppression test (LDDST). The test principle is that administration of an exogenous glucocorticoid will suppress cortisol production by a negative feedback signal to the pituitary gland in normal dogs, but not in dogs with Cushing's syndrome.<sup>23</sup> A third screening test is the ACTH-stimulation test, but due to its low sensitivity, this test is being increasingly abandoned in veterinary endocrinology.<sup>8</sup>

Once Cushing's syndrome is confirmed based on a screening test, differentiation is needed to distinguish between PDH and ATH since this has consequences for the choice of therapy. In some cases the LDDST allows differentiation between PDH and ATH. However, some pituitary tumors are "dexamethasone"-resistant and will yield LDDST results comparable to ATH.<sup>8</sup> The UCCR can also be coupled to an oral high dose dexamethasone suppression test. This test has the same limitations as the LDDST for differentiating ATH from PDH. Furthermore, plasma ACTH can be measured to distinguish between PDH (high concentration) and ATH (low concentration).<sup>3</sup>

When endocrine testing confirms PDH, the pituitary gland can be visualized using computed tomography or magnetic resonance imaging. This is necessary when hypophysectomy or pituitary irradiation are considered for therapy.<sup>3</sup> An adrenal tumor can be detected with abdominal ultrasonography, indicating enlargement of the affected adrenal gland and atrophy of the contralateral gland in ATH. In dogs with PDH, both adrenal glands

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usually have a normal size or are bilaterally enlarged, but in some cases ultrasonography indicates unequivocal asymmetry. However, in a recent study a measurement cut-off was determined that may overcome this problem, allowing ultrasonographic distinction between ATH and PDH with improved sensitivity and specificity.<sup>24</sup>

### **1.2.3 Treatment**

In dogs with PDH, treatment of choice is *transsphenoidal hypophysectomy* because it eliminates the source of hypercortisolism, i.e. the pituitary tumor causing excessive ACTH secretion (Figure 5). However, it must be kept in mind that this complex procedure has a steep learning curve for the surgeon. When performed by an experienced surgeon, efficacy is high, although survival and disease free period is higher in dogs with non-enlarged than with enlarged pituitary glands.<sup>25,26</sup> Post-operatively, lifelong supplementation with thyroxine and cortisone-acetate, and transient administration of desmopressin is needed.<sup>27</sup> Post-operative follow-up includes measurement of UCCR and total thyroxine.<sup>25</sup>

When hypophysectomy is not possible, *medical treatment* directed at inhibition of cortisol synthesis by the adrenals is an option. In the past, the adrenal cortex was selectively or non-selectively destroyed with *o,p'-DDD (mitotane)*, but since the registration of *trilostane*, its main use is limited to treatment of adrenal tumors.<sup>28</sup> Trilostane is a reversible inhibitor of the 3- $\beta$ -hydroxysteroid-dehydrogenase/isomerase system, which is necessary for cortisol synthesis. Starting dose is 2 mg/kg once daily, or 1 mg/kg twice daily in patients where short duration of action is suspected.<sup>29,30</sup> Trilostane effectively controls clinical signs of hypercortisolism, with a median survival time of 662 days (once daily) and 900 days (twice daily), with a negative correlation between survival time and age and bodyweight at diagnosis.<sup>28,31-33</sup> Optimal dosage and treatment efficacy are assessed based on clinical signs, ACTH-stimulation tests, measurement of sodium and potassium and ideally also endogenous ACTH.<sup>27</sup> Mild to moderate transient hyperkalemia can be observed during trilostane treatment. Increased ACTH and severe electrolyte abnormalities can indicate trilostane overdose, which may potentially cause life threatening hypocortisolism and mineralocorticoid deficiency.<sup>27,31,34</sup>

In dogs with a resectable adrenal gland tumor and without metastasis, *surgical removal* of the tumor is recommended. Most veterinary surgeons prefer a celiotomy, because it allows better exposure of the tumor and of possible tumor thrombi in the caudal vena cava,



but as in human medicine, laparoscopic adrenalectomy may become the treatment of choice.<sup>35,36</sup> Complication rate and mortality in the perioperative period are high, but patients that survive the 1- to 4-week post-operative period have a good life expectancy.<sup>8,37</sup>

In poor surgical candidates or patients with metastasis or a nonresectable tumor, adrenal gland tumors can be treated *medically* with the adrenocorticolytic drug *o,p'-DDD* (*mitotane*). Aim of treatment is to completely destroy the adrenocortical tissue and provide substitution therapy with cortisone acetate, fludrocortisone acetate and sodium chloride. The treatment protocol is described elsewhere.<sup>3</sup> Measurements of plasma sodium and potassium and ACTH-stimulation testing or UCCRs can be used to monitor response to therapy or possible recurrence.<sup>3</sup> Mitotane is potentially toxic to humans and should not be used in households with pregnant women or little children.

*Trilostane* can also be used as an adrenocorticostatic drug in cases where mitotane is not an option. However, information on treatment of adrenal tumors with trilostane is limited to one case series and some case-reports.<sup>38-40</sup>

**Figure 5. Female neutered, 8.5 years old Boxer with PDH (A) before and (B) six months after transsphenoidal hypophysectomy.** Note the difference in posture and improved haircoat. Pictures courtesy of Joop Fama, Utrecht University.



In the next section, focus will be on effects of glucocorticoids on renal function and, most importantly, the lack of information about renal consequences of Cushing's syndrome in human and canine patients.

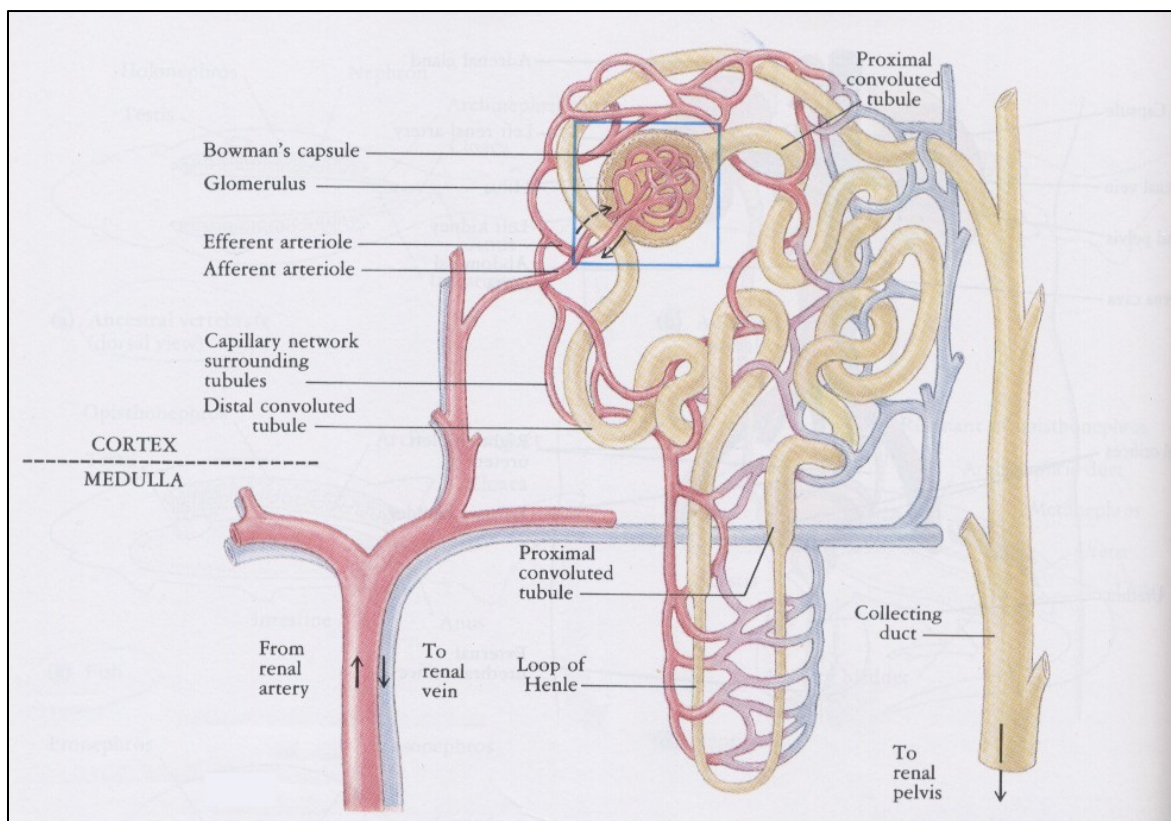
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### 1.3 Cushing's syndrome, glucocorticoids and the kidney

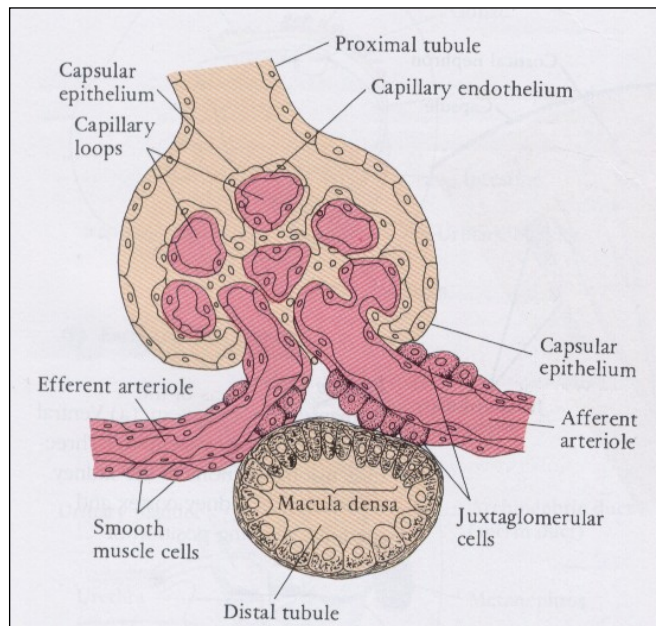
*Adapted from:* Smets P.M.Y., Meyer E., Maddens, B.E.J. and Daminet S. (2010). Cushing's syndrome, glucocorticoids and the kidney. *General and Comparative Endocrinology* 169: 1–10.

The functional unit of the kidney is the nephron, which consists of the renal corpuscle or glomerulus and the tubuli (Figure 6). The glomerulus contains a capillary network with a fenestrated endothelium, surrounded by the glomerular basement membrane and visceral epithelial cells (podocytes). These three components form the glomerular barrier through which blood is filtered to form the ultrafiltrate (Figure 7).<sup>41</sup> The glomerular barrier is charge and size selective and under physiological circumstances it retains cellular components and intermediate to high molecular weight (IMW, HMW) proteins.<sup>41</sup> Primary ultrafiltrate arrives in Bowman's capsule and flows to the tubular lumen. In the tubuli, some of the filtered substances are reabsorbed, while other plasma components are secreted into the tubular lumen. Passing through different segments of the tubuli, the tubular fluid is altered and concentrated, which eventually leads to production of urine. Normal urine is practically devoid of proteins.

**Figure 6. The nephron.** From Dorit RL, Walker WF and Barnes RD, 1991. *Zoology*, p. 356.



**Figure 7: The renal corpuscle.** Adapted from Dorit RL, Walker WF, Barnes RD, 1991. *Zoology*, p. 356.



Because of its similarity with the syndrome in humans and its high estimated incidence compared to the low incidence in people, canine Cushing's syndrome is an interesting animal model for its human counterpart.<sup>42</sup> Surprisingly, very little information exists on renal consequences of Cushing's syndrome in people as well as in dogs, while this could have important consequences for long-term outcome of this disease and patient well-being.

## CHAPTER I: *General introduction*

### *1.3.1 Vascular and hemodynamic changes*

Hypertension is a common complication of Cushing's syndrome in humans, with a prevalence of approximately 55 to 80% in adult patients and 50% in children and adolescents.<sup>43-45</sup> Likewise, systemic hypertension occurs in 59 to 86% of canine Cushing's patients.<sup>46-49</sup> Glucocorticoid (GC)-induced hypertension results from many interacting pathophysiological pathways, which may ultimately lead to an increased cardiac output (CO), total peripheral resistance (TPR) and renal vascular resistance (RVR).<sup>50</sup> Dexamethasone treatment raised blood pressure and TPR, without increase of CO in humans, and CO was actually decreased in dogs.<sup>51,52</sup>

Glucocorticoids affect vascular tone. Patients with Cushing's syndrome show increased sensitivity to angiotensin II and elevated plasma levels of the potent vasoconstrictor endothelin-1.<sup>53,54</sup> Increased diastolic and mean arterial pressure responses to noradrenaline have been demonstrated in eight human patients with Cushing's syndrome and in eight dogs with experimentally induced Cushing's syndrome.<sup>55,56</sup> Glucocorticoids seem to downregulate the expression of the Na<sup>+</sup>/Ca<sup>2+</sup>-exchanger in vascular smooth muscle cells, which causes an increased intracellular Ca<sup>2+</sup> concentration and vasoconstriction.<sup>57</sup> In cortisol-induced hypertension in humans, the serum erythropoietin concentration was increased and changes in blood pressure were correlated with changes in erythropoietin concentration. Erythropoietin is a direct vasoconstrictor, but its primary or bystander role in GC hypertension remains unclear.<sup>58</sup>

Additionally, GCs may also impair vasodilator activity. There is substantial evidence for interactions between GCs and the nitric oxide system in humans as well as rats.<sup>59-61</sup> In addition, GCs inhibited production of other vasodilating agents, such as prostacyclin, prostaglandin E2 and kallikrein.<sup>62</sup>

Glucocorticoids are also associated with occlusive vascular disease in humans, which occurs through a myriad of effects on vascular smooth muscle, endothelial cells, myocardium and macrophages, as well as their link with obesity, hypertension, dyslipidemia and insulin resistance.<sup>63</sup> Patients with Cushing's syndrome may have abnormal carotid intima thickness that persists after removal of the tumor causing GC excess.<sup>64,65</sup> Occlusive vascular disease is rare in canine patients, and dogs with atherosclerosis were not more likely to have concurrent hypercortisolism than dogs that did not have atherosclerosis.<sup>66</sup>

### ***1.3.2 Glomerular filtration rate***

The rate of ultrafiltrate formation through the glomerular barrier is the *glomerular filtration rate* (GFR). It depends on four determinants: glomerular blood flow, hydrostatic pressure gradient, plasma oncotic pressure and the glomerular ultrafiltration coefficient.<sup>67</sup> The ultrafiltration coefficient is the product of the glomerular barrier permeability and its surface area.<sup>41</sup> Increases in the first three determinants and a decrease in plasma oncotic pressure will lead to a rise in GFR.

The above mentioned GC-induced vascular and hemodynamic changes also influence renal vasculature, renal blood flow (RBF) and GFR as summarized in Figure 8. Short term administration of ACTH or GCs increases GFR in humans, rats, sheep and dogs.<sup>68-71</sup> Several hypotheses have been postulated to explain the increase in GFR.

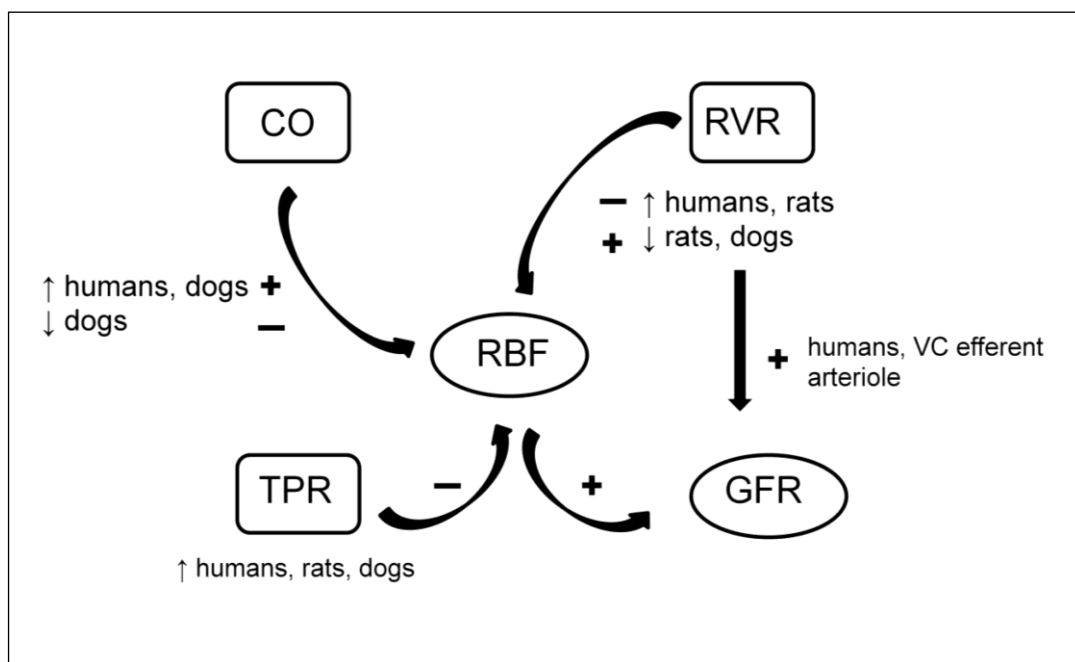
One factor that might contribute to the increased GFR is an increased RBF. In dogs, rats and sheep, GCs increase RBF, but this outcome is not always present in humans.<sup>71-75</sup> In ACTH-induced hypertensive rats and rats treated with methylprednisolone, GFR and RBF were increased, which may have been due to nitric oxide-mediated vasodilation of the pre- and post-glomerular arterioli.<sup>72,76</sup> In dogs treated with dexamethasone, RBF increased significantly. This alteration was accompanied by a decrease in RVR, which suggested that dexamethasone may have opposite effects on systemic and renal vasculature.<sup>75</sup> In the only study on RVR in dogs with spontaneous Cushing's syndrome, renal resistive index and pulsatility index were calculated as indices of RVR using Doppler ultrasound. Only 2 out of 12 dogs had higher indices, suggesting RVR may not be increased in dogs with Cushing's syndrome.<sup>49</sup>

The catabolic effects of GCs increase plasma amino acid levels, which may be another mechanism leading to an increased GFR. Indeed, the ingestion of a protein-rich meal not only increases circulating amino acids, but also RBF and GFR in humans and dogs.<sup>77,78</sup> Intravenous infusion of plasma to dogs caused hyperproteinemia, which led to a rise in GFR due to renal vasodilation, a higher RBF, either no change or a moderate increase in glomerular hydrostatic pressure and probably an increase in the glomerular ultrafiltration coefficient.<sup>79</sup>

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**Figure 8. Glucocorticoid-induced vascular and hemodynamic changes influencing renal vasculature, RBF and GFR.**

CO, cardiac output; RVR, renal vascular resistance; RBF, renal blood flow, TPR, total peripheral resistance; GFR, glomerular filtration rate; VC, vasoconstriction; ↑, increased; ↓, decreased; +, positive influence; -, negative influence.



Although acute effects of synthetic or endogenous GCs increase the GFR in laboratory animals, dogs and humans, long-term effects of Cushing's syndrome in humans may decrease GFR as shown by Haentjens and coworkers.<sup>80</sup> Most of the patients included in the latter study had been cured of Cushing's syndrome, and had a decreased GFR compared to matched controls. Permanent alterations of vessel remodeling due to the chronic endogenous state of excess cortisol may have contributed to a lower GFR in these patients.<sup>80</sup>

*Serum creatinine* (screat) is often used as an indirect measurement of GFR, although it has several limitations. Because muscle mass is the main source of serum creatinine and urinary creatinine elimination is constant over time, diseases affecting muscle mass influence the screat level.<sup>81,82</sup> Both muscular atrophy and truncal obesity are features of Cushing's syndrome in humans and dogs, and they have been shown to result in a decreased creatinine production rate.<sup>83</sup>

In people with Cushing's syndrome, both increased and reference range screat levels have been reported.<sup>80,83,84</sup> Patients receiving prednisone showed a rise in plasma creatinine

and urinary creatinine excretion, which was probably due to a catabolic effect.<sup>85</sup> Screat in dogs with Cushing's syndrome was usually within or lower than the reference range.<sup>8,86</sup>

### **1.3.3 Proteinuria**

In normal urine, only small amounts of protein are present. Proteinuria occurs in 44 to 75% of dogs with Cushing's syndrome and is a sign of kidney dysfunction.<sup>46,47</sup> It can be caused by glomerular dysfunction, allowing leakage of excess proteins into the ultrafiltrate, and/or by tubular dysfunction, leading to impaired reabsorption of filtered proteins.<sup>87</sup> There is evidence that protein overload in the ultrafiltrate may be toxic to the tubular cells and trigger pathways that cause tubulo-interstitial injury.<sup>88</sup> Urinary albumin (ALB) is increased in humans, dogs and rats treated with glucocorticoids as well as canine Cushing patients.<sup>89-93</sup> In dogs treated with hydrocortisone, hypertension was suggested as a possible mechanism contributing to proteinuria, along with the known hemodynamic alterations leading to increased renal blood flow and GFR.<sup>75,91</sup> In one study in people, increased urinary albumin excretion in patients with active Cushing's syndrome was suggested to be associated with disturbances in lipid metabolism.<sup>94</sup> Even with a normal permselectivity of the glomerular barrier, excessive binding of serum albumin to free fatty acids may lead to increased urinary albumin excretion.<sup>95</sup> Activation of the protein kinase C pathway by excessive plasma free fatty acids in renal endothelial cells may also contribute to renal injury and proteinuria.<sup>96-98</sup>

### **1.3.4 Tubular changes**

#### *Electrolyte handling*

In states of excess cortisol, 11 $\beta$ -HSD2 enzyme activity (see paragraph 1.1, p.7) is saturated and cortisol is allowed to bind to mineralocorticoid receptors in renal distal tubular and collecting duct cells.<sup>99</sup> Mineralocorticoid receptors regulate expression of the epithelial sodium channel and the sodium-potassium ATP-ase pump. Thus, receptor ligand binding causes increased *sodium* reabsorption.<sup>100-102</sup> This finding was demonstrated in dogs treated with methylprednisolone which had very high filtered sodium loads but only mild increases in urinary sodium-excretion.<sup>74</sup> Mean urinary sodium levels in human patients with active Cushing's syndrome were lower compared to cured patients and healthy controls.<sup>84</sup> The mineralocorticoid effect of cortisol does not seem to influence renal *potassium* handling, except in human and canine patients with ectopic secretion of ACTH who often have hypokalemia.<sup>14,15,103</sup> In the syndrome of ectopic ACTH secretion, ACTH may inhibit 11 $\beta$ -

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HSD2 enzyme activity either directly or by stimulating an inhibitory adrenal product, in addition to enzyme saturation by excess cortisol.<sup>103</sup> In most people and dogs with Cushing's syndrome, plasma sodium and potassium concentrations were within reference ranges.<sup>48,104,105</sup>

Glucocorticoids decrease renal *calcium* ( $\text{Ca}^{2+}$ ) absorption. Mineralocorticoid receptor-mediated activation of the epithelial sodium channel causes depolarization of the cell membrane, which inhibits  $\text{Ca}^{2+}$ -entry and  $\text{Ca}^{2+}$ -efflux via the sodium/ $\text{Ca}^{2+}$ -exchanger and  $\text{Ca}^{2+}$ -ATPase in distal tubular cells, leading to an increased fractional  $\text{Ca}^{2+}$  excretion.<sup>106</sup> Hypercalciuria is present in 86% of people with Cushing's disease, and dogs with Cushing's syndrome are 10 times more likely to have calcium-containing uroliths than control dogs.<sup>84,107</sup> Despite hypercalciuria, plasma ionized  $\text{Ca}^{2+}$  was normal in people and dogs with hypercortisolism compared with control subjects.<sup>86,108</sup>

Human patients with Cushing's syndrome have decreased renal *phosphate* reabsorption and hypophosphatemia whereas canine patients have elevated serum phosphorus. Dogs with Cushing's syndrome have a marked increase in basal parathyroid hormone. In comparison, humans only show a mild elevation in parathyroid hormone, if any. It has been suggested that increased parathyroid hormone levels in dogs with Cushing's syndrome may be a compensatory response to increased  $\text{Ca}^{2+}$  loss and/or increased phosphate concentration. After treatment, serum ionized  $\text{Ca}^{2+}$  and phosphorus rise in humans, but they decrease in dogs. These findings illustrate marked differences in calcium and phosphorus metabolism between humans and dogs.<sup>86,108,109</sup>

### *Urine concentrating ability*

In addition to electrolyte handling, GCs are also involved in renal water excretion. Studies from the 1950s have demonstrated impaired water load excretion in adrenocortical insufficiency and increased diuresis with cortisone administration in people and dogs.<sup>110-112</sup> Although both humans and dogs with endogenous Cushing's syndrome can present with polyuria as a clinical sign, this feature is most pronounced in dogs and is one of the principal symptoms.<sup>8,113,114</sup> Dilute urine with a USG < 1.020 occurs in 85% of dogs with Cushing's syndrome.<sup>8</sup> Glucocorticoid effects on diuresis are mediated by their dual interaction with arginine vasopressin (AVP). Excess cortisol disturbs osmoregulation of AVP release at the level of the hypothalamus and/or neurohypophysis, and causes resistance to AVP action in the kidneys.<sup>113-116</sup> Stimuli that increase AVP, such as water deprivation or administration of



desmopressin, only caused marginal increases in urine osmolality in humans and dogs with hypercortisolism.<sup>113,114</sup> Polyuria in canine hypercortisolism and hyperaldosteronism has been suggested to be caused by AVP resistance and increased osmotic threshold of AVP release.<sup>113,117,118</sup>

Urinary dilution and concentration is regulated by water channels or aquaporins (AQP), urea and ion transporters, some of which are under AVP control.<sup>119</sup> In dexamethasone treated rats, AQP1 and AQP3 expression was upregulated, whereas AQP2 expression was not significantly different compared with control rats. If alterations in these water channels contributed to the urinary concentration defect, their expression should have been downregulated.<sup>120</sup> However, expression of urea transporters was consistently decreased in dexamethasone treated rats or adrenalectomized rats with GC replacement.<sup>120,121</sup> Therefore, the authors concluded that decreased urea accumulation caused lower interstitial osmolality in the inner renal medulla through downregulation of urea transporters, which contributed to the impaired urine concentrating ability in the excess GC state.<sup>120</sup> Effects of ACTH, endogenous or exogenous GCs on renal glomerular and tubular function are summarized in Table 2.

**Table 2. Effects of ACTH, endogenous (CS) or exogenous GCs (DEX, MP) on renal glomerular and tubular function.** ACTH, adrenocorticotrophic hormone; CS, endogenous Cushing’s syndrome; DEX, dexamethasone; MP, methylprednisolone; ↑, increased; ↓ decreased.

	↑ or ↓	Species	ACTH, CS, DEX, MP
<b>Glomerular function:</b>			
GFR	acute ↑ chronic ↓	humans, rats, sheep, dogs humans	ACTH, DEX, MP CS
Proteinuria	↑	humans, rats, dogs	DEX, CS, MP
<b>Tubular function:</b>			
Na <sup>+</sup> reabsorption	↑	humans, rats, dogs	ACTH, CS, DEX, MP
Ca <sup>2+</sup> reabsorption	↓	humans, dogs	CS
Phosphate reabsorption	↓	humans	CS
Urine concentrating ability	↓	humans, rats, dogs	ACTH, DEX, CS

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The vast majority of information on the interaction between GCs and the kidney was generated after administration of ACTH or exogenous GCs. Only one study has described GFR in human Cushing patients and there are no studies evaluating both glomerular and tubular function.<sup>122</sup> Two studies in the nineties described hypertension and proteinuria in dogs with Cushing's syndrome.<sup>46,47</sup> However, the cut-off for proteinuria was higher than currently defined, GFR measurement and long-term follow-up post-treatment was not available and dogs were treated with mitotane, while nowadays trilostane is used for medical treatment. Therefore, evaluation of renal consequences of this endocrine disorder is needed in human and canine patients, because they may have implications for the general well-being of the patient.

In the last section of this chapter we will discuss different methods for evaluation of renal function in dogs.

**1.4 Evaluation of renal function**

**1.4.1 Routine renal markers**

Kidney function is routinely assessed by measurement of serum or plasma creat and urea. The classification system for canine and feline chronic kidney disease (CKD), developed by the International Renal Interest Society (IRIS) is based on measurement of screat (Table 3). Dogs or cats with stable renal disease are assigned to stage I to IV according to their screat concentration, with substages based on systolic blood pressure (SBP) and proteinuria. Patients are sub-staged by SBP according to the degree of risk of end-organ damage or presence of end-organ damage or complications. In dogs, a SBP < 150 mmHg is suggested to cause minimal risk, a SBP between 150-159 mmHg low risk, between 160-179 mmHg moderate risk, and  $\geq 180$  mmHg high risk. Canine patients are considered non-proteinuric when their urinary-protein-to-creatinine ratio (UPC) is < 0.2, borderline proteinuric when their UPC is > 0.2 but < 0.5, and proteinuric when their UPC is > 0.5. Based on this staging proces, guidelines for treatment and follow-up are recommended.<sup>123</sup>

**Table 3. International Renal Interest Society (IRIS) staging of CKD in dogs, based on screat concentration.**<sup>123</sup>

CKD stage	screat ( $\mu\text{mol/l}$ )	Comments
I	< 125	Non-azotemic Some other renal abnormality present e.g.: <ul style="list-style-type: none"> <li>- inadequate concentrating ability without identifiable non-renal cause</li> <li>- abnormal renal palpation and/or abnormal renal imaging findings</li> <li>- proteinuria of renal origin</li> <li>- abnormal renal biopsy results</li> </ul>
II	125-179	Mild renal azotemia Clinical signs usually mild or absent
III	180-439	Moderate renal azotemia Many systemic clinical signs may be present
IV	> 440	Severe renal azotemia Many extra-renal clinical signs present
<i>Note: screat levels apply to average sized dogs – extreme sizes may cause variation</i>		

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However, creatinine and urea allow detection of kidney dysfunction only when at least 75% of renal functional mass is lost.<sup>124</sup> Hence there is a need for markers that allow early detection of renal dysfunction. Glomerular filtration rate is considered the best overall index of renal function. In human medicine, certain urinary markers allow early and site-specific indication of renal dysfunction.

### *1.4.2 Measurement of glomerular filtration rate*

Measurement of GFR is considered a more sensitive index of renal function, because it allows detection of a decreased glomerular function before development of azotemia.<sup>125,126</sup> Determination of GFR is based on the concept of *clearance*, or the rate with which plasma is cleared of a substance by the kidney. Clearance can be determined using both urinary and plasma data of a certain marker (*urinary clearance*) or using only plasma concentration versus time data (*plasma clearance*).<sup>127</sup> Urinary clearance of a marker is calculated with the following formula:  $Cl_{\text{urinary}} = (U \times C_u)/C_p$ , where  $U$  = urine flow in mL/min,  $C_u$  = marker concentration in urine in mg/mL, and  $C_p$  = marker concentration in plasma in mg/mL. Plasma clearance is calculated by the formula:  $Cl_{\text{plasma}} = D/AUC$  where  $D$  = dose of the marker and  $AUC$  = area under the plasma concentration versus time curve. The AUC is calculated based on an appropriate pharmacokinetic model.<sup>125,127</sup>

An ideal filtration marker is not protein bound, does not enter the red blood cells, is homogeneously distributed in bodywater, and is solely excreted through the kidney by glomerular filtration, without tubular reabsorption or secretion. Additionally, it must not be toxic or in itself alter GFR.<sup>125,127</sup> The fructose polymer inulin approaches these prerequisites and urinary inulin clearance is considered the gold standard for determination of GFR.<sup>128,129</sup>

However, urinary clearance tests are impractical in veterinary practice because they require accurate collection of timed urine samples. There is also a risk of urinary tract infection. Plasma clearance methods using a single bolus injection of a marker followed by timed blood sampling are therefore more attractive.<sup>125,127</sup>

Several markers have been evaluated for measurement of plasma clearance in dogs, such as radiolabeled filtration markers, exogenous creatinine and iodinated contrast media.<sup>127</sup>  $^{125}\text{I}$ - or  $^{131}\text{I}$ -iothalamate,  $^{51}\text{Cr}$ -ethylenediaminetetraacetic acid ( $^{51}\text{Cr}$ -EDTA) and  $^{99\text{m}}\text{Tc}$ -diethylenetriaminepentaacetic acid ( $^{99\text{m}}\text{Tc}$ -DTPA) have all been widely used as GFR markers in the dog.<sup>130-132</sup> Important disadvantages are the need for a specialised nuclear medicine

laboratory and the safety hazard related to working with radionuclides. *Plasma clearance of exogenous creatinine* was described to be an accurate indicator of GFR in healthy and renal impaired dogs, correlating well with urinary inulin clearance.<sup>125</sup> Weak tubular secretion of creatinine exists in the dog, but is of negligible significance for GFR determination.<sup>125,133</sup> Advantages are that plasma creatinine can be measured with inexpensive, rapid and widely available assays which require only a small volume of blood. Potential drawbacks for use in practice is that there is currently no commercially available injectable creatinine preparation.<sup>125</sup> Iohexol is a iodinated compound of a contrast medium that can also be used to measure GFR in dogs.<sup>134</sup> *Plasma iohexol clearance* is often considered as a reference method in humans and correlates well with urinary clearance of inulin and creatinine in dogs.<sup>135-138</sup> Iohexol is stable in frozen samples, safe, even in patients with decreased renal function, and commercially available. However, a disadvantage is that iohexol analysis with high-performance liquid chromatography requires a specialised laboratory.<sup>127</sup>

Plasma exogenous creatinine clearance has been used to determined GFR in dogs with spontaneous and experimentally induced hypothyroidism, dogs with leishmaniasis and dogs with CKD.<sup>139-142</sup> Plasma iohexol clearance was measured in dogs with heart failure, experimentally induced renal failure and pyometra.<sup>135,143,144</sup> To date, there are no studies investigating GFR in dogs with Cushing's syndrome.

### **1.4.3 Urinary biomarkers**

*Adapted from:* Maddens B.E.J., Daminet S., Smets P.M.Y. and Meyer E. (2011). Urinary biomarkers for kidney dysfunction in dogs. *Journal of Veterinary Internal Medicine*, in preparation.

Biomarkers are defined as biological parameters that can be objectively measured, and act as indicators of normal or pathological processes, or of the response to intervention. The ideal biomarker for kidney injury or dysfunction should be able to (1) detect kidney dysfunction in an early stage, (2) localize the site of kidney dysfunction (e.g. glomerular versus tubular), (3) differentiate renal injury from pre-, post-, and non-renal injury, (4) predict the severity of kidney injury and (5) allow monitoring of effects of intervention.<sup>145</sup> To be clinically applicable, the biomarker should be accurate, easy to measure and non-invasive. Increased concentrations of serum markers are only useful in diagnosis of the primary renal insult if they are known to originate specifically from the kidney.<sup>146</sup> In contrast, urine may be

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a more promising biological fluid to identify the earliest biomarkers of kidney dysfunction because of its proximity to the kidney, and its easy accessibility.<sup>147</sup>

### 1.4.3.1 Glomerular markers

#### *Albumin and other intermediate molecular weight proteins*

*Albumin* (ALB, 69 kDa) is one of the first candidate urinary markers that has been studied in dogs. Microalbuminuria is defined as a urinary albumin concentration of 1-30 mg/dl and persistent microalbuminuria is considered the mildest form of renal proteinuria.<sup>148</sup> Several studies suggest that microalbuminuria may be an early indicator of glomerular disease in dogs.<sup>149,150</sup> However, increased urinary (u) concentrations of ALB were also observed in dogs with non-renal diseases, raising questions concerning specificity for diagnosis of renal disease.<sup>151,152</sup> Furthermore, its potential for early detection of kidney dysfunction in nonazotemic dogs has recently been questioned.<sup>153</sup> Presence of uALB can be related to both glomerular and tubular dysfunction, although large uALB amounts generally reflect altered glomerular permselectivity.<sup>148</sup>

Other intermediate molecular weight proteins (IMW) such as *vitamin D-binding protein* (56 kDa) and *transthyretin* (55 kDa) have been reported to reflect proximal tubular dysfunction. Urinary *transferrin* (76 kDa) was demonstrated to be indicative of glomerular proteinuria.<sup>154</sup> Increased urinary amounts of these proteins were observed by western blot analysis of samples from dogs with chronic renal failure and X-linked hereditary nephropathy (XLHN).<sup>155,156</sup> However, these proteins remain to be quantitatively analyzed and their potential as urinary biomarker for kidney dysfunction in dogs is unknown.

#### *Immunoglobulin G and other urinary high molecular weight proteins*

*Immunoglobulin G* (IgG, 150 kDa) is produced by activated B-lymphocytes (plasma cells) and circulates in plasma as part of the humoral immunity. Presence of this high molecular weight (HMW) protein in urine has been linked to altered glomerular permselectivity and glomerular lesions in humans.<sup>154</sup> Urinary IgG (uIgG) was increased in dogs with CKD,<sup>155</sup> XLHN,<sup>156</sup> leishmaniasis,<sup>157,158</sup> pyometra,<sup>159</sup> and leptospirosis.<sup>160</sup> Urinary IgG was increased compared to age-matched healthy control dogs and present prior to azotemia, thereby suggesting a role for early detection of altered glomerular permselectivity.<sup>156</sup>

In analogy with IgG, urinary *IgA* was found to be increased in dogs with leishmaniasis,<sup>158,161</sup> leptospirosis,<sup>162</sup> and pyometra<sup>159</sup> compared to healthy dogs. However, quantification of IgA was not performed and results were based on gel electrophoreses of urine. Recently, urinary C-reactive protein (CRP, 115 kDa) was demonstrated to be increased in urine of dogs with leptospirosis.<sup>162</sup>

### 1.4.3.2 Tubular markers

#### Retinol-binding protein and other urinary low molecular weight proteins

*Retinol-binding protein* (RBP, 21 kDa) is a low molecular weight (LMW) plasma protein synthesized in the liver and is the major carrier of retinol.<sup>163</sup> Uncomplexed RBP is freely filtered by the glomeruli and efficiently reabsorbed by megalin-mediated endocytosis in the proximal tubules.<sup>164</sup> Therefore, urine of healthy dogs is normally almost devoid of RBP and increased urinary RBP (uRBP) indicates impaired tubular reabsorption.<sup>165,166</sup> Higher concentrations of uRBP were observed in dogs with CKD and urolithiasis compared with healthy controls.<sup>155,167,168</sup> The potential of uRBP to diagnose kidney dysfunction at an early stage has recently been evaluated in two studies. In one study, increased uRBP was detected before the onset of azotemia in dogs with XLHN.<sup>169</sup> In addition, results of the latter study support the correlation of uRBP with progression of CKD. In contrast, a second study reported uRBP concentrations not to be significantly associated with plasma creatinine clearance in non-azotemic dogs with CKD. Consequently, the authors concluded that uRBP might not be useful for detection of a mildly decreased GFR in early stages of renal disease.<sup>153</sup> Although to date uRBP seems to be a promising marker for proximal tubular dysfunction in dogs, further research is warranted.

Other urinary LMW proteins such as  $\alpha_1$ -microglobulin and  $\beta_2$ -microglobulin have been intensively studied as tubular markers in human kidney diseases.<sup>170,171</sup> Only one study demonstrated these proteins in urine of dogs with XLHN and they were found to increase with renal disease progression.<sup>156</sup>

#### Tubular enzymes

*N-acetyl- $\beta$ -D-glucosaminidase* (NAG, 150 kDa), *alkaline phosphatase* (AP),  *$\gamma$ -glutamyl transferase* (GGT), *alanine aminopeptidase* (AAP), and *lactate dehydrogenase* (LDH) are enzymes primarily located in the lysosomes (NAG, AP), brush border (GGT, AAP) or cytoplasm (LDH) of proximal tubular cells. These tubular enzymes are excreted in

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urine after tubular dysfunction or damage.<sup>172,173</sup> The magnitude of urinary NAG (uNAG), AP, and GGT concentrations was correlated with severity of lesions in proximal tubular cells in dogs with pyometra.<sup>174</sup> Furthermore, early onset of acute renal damage was accurately reflected by two- to threefold increases of urinary NAG and GGT in dogs with gentamicin-induced nephrotoxicosis.<sup>175,176</sup> In contrast, uNAG-, GGT- and LDH-to-urinary creatinine ratios were higher but not significantly increased compared to baseline after cisplatin-induced acute kidney injury in dogs, despite presence of azotemia and histological evidence of tubular injury.<sup>177</sup> No explanation for this finding was given, although high inter-individual variation may have played a role. Increased uNAG and GGT concentrations compared to healthy controls were shown in dogs with CKD.<sup>178</sup> Urinary NAG activity was increased in dogs with pyelonephritis when compared to patients with uncomplicated lower urinary tract infections.<sup>179</sup> So far, uNAG is one of the most studied tubular markers in dogs.

**Table 4. Overview of urinary biomarkers for kidney dysfunction in dogs.**

Urinary biomarker	Nephron segment	Clinical conditions in which the biomarker has been studied in dogs
<b>IMW/HMW proteins</b>		
ALB	Glomerulus and proximal tubule	CKD, acute renal failure, hypercortisolism, diabetes mellitus, critical care, anesthesia, urinary tract inflammation,
IgG and IgA	Glomerulus	Leishmaniasis, leptospirosis, pyometra, hypercortisolism, diabetes mellitus, X-Linked Hereditary Nephropathy (XLHN)
<b>LMW proteins</b>		
RBP	Proximal tubule	CKD, XLHN, urolithiasis
$\beta_2$ -microglobulin	Proximal tubule	XLHN
$\alpha_1$ -microglobulin	Proximal tubule	XLHN
<b>Tubular enzymes</b>		
NAG	Proximal > distal tubule	Aminoglycoside-nephrotoxicosis, maleate administration, pyometra, hypercortisolism, diabetes mellitus, CKD, acute renal failure, leishmaniasis, urinary tract inflammation
GGT	Proximal tubule	
AAP	Proximal tubule	
AP	Proximal > distal tubule	



## **1.5 Conclusion**

There is an extensive connection between GCs and renal function. Excess cortisol in Cushing's syndrome may have detrimental effects on renal function by inducing vascular and hemodynamic alterations as well as glomerular and tubular dysfunction. In addition, exogenous GCs are widely used in human and veterinary medicine for a myriad of indications.

Because of its similarity with the syndrome in humans and its high estimated incidence compared to the low incidence in people, canine Cushing's syndrome is an interesting animal model for its human counterpart.

Studies evaluating renal consequences of Cushing's syndrome in human and canine patients are scarce. Better characterization of renal involvement may have implications for therapy as well as the general well-being of the canine and human Cushing patient.

Since routine markers such as creatinine and urea are insensitive indicators of renal function, there is a need for sensitive markers that allow early detection of renal dysfunction. Measurement of GFR is considered the best index of overall renal function, whereas urinary markers may allow early and site-specific indication of renal dysfunction. Only few studies have described the use of urinary markers in veterinary medicine.

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## **CHAPTER II:**

### **SCIENTIFIC AIMS**

## CHAPTER II: *Scientific aims*

Excess cortisol in Cushing's syndrome may affect renal function by inducing vascular and hemodynamic alterations as well as glomerular and tubular dysfunction. Fifty-nine to 86% of canine Cushing patients have hypertension and 44 to 75% have proteinuria. However, studies evaluating renal consequences of Cushing's syndrome or exogenous GCs are scarce in human as well as in veterinary medicine.

Therefore, the general aim of this thesis is to assess effects of Cushing's syndrome on renal function in dogs. Routine renal markers, namely serum creatinine and urea, will only be increased when 75% of renal function is already compromised. Hence, more sensitive methods, such as measurement of glomerular filtration rate (GFR) and urinary markers are necessary to detect renal dysfunction at an earlier stage.

Specific objectives of the thesis are:

1. To evaluate sampling methods and storage conditions of canine urine samples for measurement of urinary albumin (uALB), retinol-binding protein (uRBP) and N-acetyl- $\beta$ -D glucosaminidase (uNAG)
2. To validate precision, accuracy and sensitivity of a commercially available uALB ELISA and uNAG colorimetric assay and to determine glomerular (uALB) and tubular (uRBP, uNAG) marker concentrations in urine samples of healthy young and aged dogs, and dogs with chronic kidney disease
3. To assess renal function in dogs with Cushing's syndrome using GFR and uALB, urinary immunoglobulin G (uIgG), uRBP and uNAG, before treatment and during a 12 month follow-up period after medical or surgical therapy of hypercortisolism
4. To evaluate renal function and morphology using GFR, urinary markers and renal biopsies in aged Beagle dogs before and after inducing hypercortisolism

## CHAPTER II: *Scientific aims*

**CHAPTER III:**  
**EFFECTS OF SAMPLING METHOD AND STORAGE**  
**CONDITIONS ON URINARY MARKERS**

**CHAPTER III: *Sampling method and storage conditions for urinary markers***



**EFFECTS OF SAMPLING METHOD AND STORAGE CONDITIONS ON URINARY  
ALBUMIN, RETINOL-BINDING PROTEIN AND N-ACETYL- $\beta$ -D-  
GLUCOSAMINIDASE CONCENTRATIONS**

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## **CHAPTER III: *Sampling method and storage conditions for urinary markers***

### **Summary**

Urinary markers for renal dysfunction are gaining interest, but effects of sampling method, storage conditions, and urinary tract inflammation or infection on these markers are unclear. Therefore, the objectives of the current study were to determine the difference in urinary albumin (uALB), urinary retinol-binding protein (uRBP), and urinary N-acetyl- $\beta$ -D-glucosaminidase (uNAG) concentrations in cystocentesis and voided samples and to investigate concentration changes after storage at  $-20^{\circ}\text{C}$  and at  $-80^{\circ}\text{C}$ . Effects of a protease inhibitor were also assessed in samples stored at  $-80^{\circ}\text{C}$  for 12 months. In a pilot experiment, influence of in vitro hematuria, pyuria, and bacteriuria on the urinary markers was evaluated. A mixed model was used to calculate mean differences and 95% confidence intervals.

Urinary ALB, uNAG, and uRBP concentrations were similar in voided and cystocentesis samples. After storage for 4 months at  $-20^{\circ}\text{C}$ , uALB concentration was not affected, and uRBP concentration showed a mild and clinically irrelevant decrease, whereas uNAG activity was significantly lower compared to fresh samples. After storage for 12 months at  $-80^{\circ}\text{C}$ , uALB and uRBP concentrations did not differ from fresh samples, but uNAG activity was severely decreased. Protease inhibitor addition did not preserve uNAG activity. Experimental hematuria, pyuria, and bacteriuria did not seem to affect urinary markers, although further research is needed.

## CHAPTER III: *Sampling method and storage conditions for urinary markers*

### **Introduction**

Early diagnosis of decreased kidney function remains a challenge in veterinary medicine because of limitations of current diagnostic tests. Traditional diagnostic markers such as serum creatinine (screat) and ureum will only be increased when already 75% of functional renal mass is lost.<sup>1</sup> Measurement of glomerular filtration rate (GFR) is a more sensitive technique, but usually requires labor-intensive and time-consuming clearance tests.<sup>2</sup> Therefore, novel urinary markers that have demonstrated their potential for early and site-specific detection of renal dysfunction in human studies are gaining interest in companion animal medicine.<sup>3-6</sup> Among others, these urinary markers include intermediate weight proteins such as albumin (ALB), low molecular weight proteins such as retinol-binding protein (RBP), and urinary enzymes such as N-acetyl- $\beta$ -D-glucosaminidase (NAG). Glomerular dysfunction causes increased filtration of ALB, whereas tubular dysfunction leads to excessive urinary excretion of RBP and NAG due to disturbed reabsorption or structural damage of tubule cells, respectively.<sup>7</sup> These markers may not only allow early diagnosis of primary renal disease, they are also able to detect subclinical renal dysfunction secondary to infectious or endocrine disorders (e.g. pyometra, leishmaniasis, hyperthyroidism, and Cushing's disease).<sup>8-11</sup>

In order to perform reliable clinical studies using urinary markers, urine samples need to be collected and stored in optimal conditions to preserve protein integrity and enzyme activity. Studies evaluating the effect of sampling method and storage conditions on determination of urinary markers are lacking in veterinary medicine and therefore mandatory. Hence, the first objective of the current study was to determine whether urinary (u) ALB, uRBP, and uNAG concentrations differ between samples collected by cystocentesis or by free catch. The second aim was to investigate whether urinary marker concentrations decrease over time depending on storage duration and freezing temperature and if a protease inhibitor (PI) cocktail preserves protein concentrations in samples stored at  $-80^{\circ}\text{C}$  for 12 months. In a pilot experiment, effects of in vitro hematuria, pyuria, and bacteriuria on urinary marker concentrations were assessed.

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### **Material and Methods**

#### *Urine samples*

The present study was performed at the Faculty of Veterinary Medicine, Ghent University (Merelbeke, Belgium), after approval by the Local Ethical Committee (number 2008/066) and written owner consent. Eleven privately owned dogs were included (2 neutered males, 5 intact females, and 4 spayed females), diseased as well as healthy, to ensure a broad range of urinary marker concentrations. The study group consisted of 3 healthy dogs, 1 dog with diabetes mellitus, 1 dog with combined diabetes mellitus and Cushing's disease, 1 dog with Cushing's disease, and 5 dogs with chronic kidney disease. Immediately after morning urine samples were obtained by ultrasound-guided cystocentesis, dogs were walked, and a voided urine sample was collected from each dog. During cystocentesis, care was taken to avoid peripheral blood contamination, and none of the samples showed macroscopic discoloration possibly leading to falsely increased uALB concentrations.<sup>12</sup>

After centrifugation (3 min,  $447 \times g$ ), a complete urinalysis (dipstick,<sup>a</sup> urine specific gravity, urine protein-to-creatinine ratio (UPC), sediment analysis, and bacterial culture) was done on cystocentesis samples. Dipstick and sediment analysis of the voided samples were also performed. The remaining supernatant was divided into aliquots of 0.5 ml and frozen at  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$ . Sufficient aliquots were frozen to avoid multiple freeze-thaw cycles during future analyses. Fifty  $\mu\text{l}$  of PI cocktail<sup>b</sup> was added to 1 ml of cystocentesis and voided urine, and 4 aliquots of each were frozen at  $-80^{\circ}\text{C}$ . One aliquot of both cystocentesis and voided samples was used immediately for measurement of uALB, uRBP, and uNAG in fresh samples.

#### *Analysis of urinary markers*

Urinary ALB, uRBP, and uNAG concentrations were measured in cystocentesis and voided samples, within 3 hr after collection (time 0:  $T_0$ ) and after 4 months of storage at  $-20^{\circ}\text{C}$  (time 4 months:  $T_{4 \text{ months}}$ ). Furthermore, urinary markers were determined in cystocentesis samples after 12 months of storage at  $-20^{\circ}\text{C}$  and at  $-80^{\circ}\text{C}$  (time 12 months:  $T_{12 \text{ months}}$ ) and in PI samples stored at  $-80^{\circ}\text{C}$ . Urinary ALB concentration was determined with a commercial canine-specific sandwich enzyme-linked immunosorbent assay (ELISA) kit,<sup>c</sup> uRBP with a human ELISA kit,<sup>d</sup> and uNAG activity using a colorimetric assay,<sup>e</sup> as previously validated and described.<sup>3,6</sup> Mean interassay coefficient of variation was 12.0% for uALB analysis, 8.5% for uRBP, and 8.1% for uNAG.<sup>3,6</sup>

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### *Effect of hematuria, pyuria, and bacteriuria*

Cystocentesis urine samples of 2 healthy dogs were used to evaluate the effect of experimental hematuria, pyuria, and bacteriuria on uALB, uRBP, and uNAG concentrations. A heparinized blood sample of 1 healthy dog was used to mimic hematuria and to isolate white blood cells. Whole blood was diluted 4,000- and 16,000-fold in the urine samples, yielding a 3+ and 1+ hematuria on dipstick analysis,<sup>f</sup> respectively. Furthermore, white blood cells were isolated after gradient centrifugation on Percoll.<sup>g,13</sup> Briefly, 2 ml of blood was layered carefully on Percoll with a density of 1.077. The tube was centrifuged at  $340 \times g$  for 30 min at room temperature. Then plasma was discarded, and the opaque band at the interface between plasma and the Percoll layer, containing the white blood cells, was aspirated with a Pasteur pipette and transferred into a conical tube. After 3 washing steps in a phosphate buffered saline solution, the white blood cells were counted.<sup>h</sup> Cytospin preparations<sup>i</sup> were stained<sup>j</sup> and showed isolated white blood cells to consist mainly of neutrophils (74%) and further of lymphocytes (21%) and monocytes (5%). White blood cells were added to the urine samples until a final concentration of approximately 80 white blood cells per  $\mu\text{l}$  was obtained, corresponding with 1+ pyuria on dipstick analysis.

For the bacteriuria experiment, *Escherichia coli* bacteria were grown overnight, and bacterial concentration was determined using a standard curve plotting the colony forming units (CFUs) as a function of the absorbance at 550 nm. Bacteria were diluted in the urine samples until a final concentration of  $10^5$  CFU per ml was obtained.

All urine samples (i.e., 1+ and 3+ hematuria, 1+ pyuria, and bacteriuria) and blank urine samples were incubated at  $38^\circ\text{C}$  for 2 hr to simulate a retention period in the bladder at body temperature. After centrifugation (3 min at  $447 \times g$ ), the supernatant was divided into aliquots of 1 ml and stored at  $-80^\circ\text{C}$  until analysis within 2 weeks. Additionally, blank urine samples without incubation at  $38^\circ\text{C}$  were also centrifuged and stored at  $-80^\circ\text{C}$  as negative controls. Urinary ALB, uRBP, and uNAG concentrations were compared between blank samples and hematuria, pyuria, and bacteriuria samples of the 2 dogs to assess the effect of red blood cell, white blood cell, and bacterial contamination.

### *Statistical analysis*

Analyses were performed with a commercial software program<sup>k</sup> using a mixed model with dog as random effect, and with collection method, storage time, freezing temperature, and addition of PI as categorical fixed effects. Urinary ALB, uRBP and uNAG concentrations were compared between cystocentesis and voided samples at  $T_0$  and at  $T_4$  months. Urinary

### **CHAPTER III: *Sampling method and storage conditions for urinary markers***

marker concentrations at T<sub>4 months</sub> and T<sub>12 months</sub> were compared to concentrations at T<sub>0</sub>. At T<sub>12 months</sub>, uALB, uRBP, and uNAG concentrations in samples stored at -80°C with and without PI were compared to each other and to T<sub>0</sub> samples. All tests were performed at 5% significance level.

Mean differences between sampling techniques, storage time, and temperature with 95% confidence intervals (CI) were derived. Equivalence between sampling techniques or between storage conditions can be claimed when the confidence interval is contained in a region of differences, which is considered to be clinically irrelevant. To calculate the mean percentage change of uALB, uRBP, and uNAG over time, a simple regression analysis was performed with difference at 2 time points as response variable and the value at earliest time point as covariate.

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#### Results

There was no statistically significant difference in uALB, uRBP, and uNAG concentrations between cystocentesis and voided urine samples at  $T_0$  or  $T_{4 \text{ months}}$  ( $P > 0.05$ ). Mean differences between cystocentesis and voided samples at  $T_0$  and  $T_{4 \text{ months}}$  are presented in Figure 1.

Differences in uALB, uRBP, and uNAG concentrations after storage at  $-20^\circ\text{C}$  and  $-80^\circ\text{C}$  are also shown in Figure 1. At  $T_{4 \text{ months}}$  after storage at  $-20^\circ\text{C}$ , uALB concentration was not significantly different from  $T_0$  ( $P > 0.05$ ), with a mean difference of 29.96 mg/l (95% CI: [-18.55; 51.48]). Urinary RBP concentration was significantly but mildly decreased ( $P = 0.02$ ), with a mean difference of 40.11  $\mu\text{g/l}$  (95% CI: [12.51; 67.71]). Urinary NAG activity was significantly lower compared to  $T_0$ , with a mean decrease of 0.61 U/l (95% CI: [0.27; 0.95];  $P = 0.005$ ). Mean percentage decrease  $\pm$  standard error (SE) was  $15 \pm 3\%$  for uRBP and  $20 \pm 11\%$  for uNAG, respectively.

At  $T_{12 \text{ months}}$  after storage at  $-20^\circ\text{C}$ , uALB and uNAG concentrations were significantly lower than at  $T_0$  ( $P = 0.006$  and  $P < 0.0001$ , respectively). Mean difference was 99.83 mg/l (95% CI: [44.25; 155.4]) and 2.87 U/l (95% CI: [2.13; 3.6]) for uALB and uNAG, respectively. For uALB, mean percentage decrease  $\pm$  SE was  $20 \pm 6\%$  and for uNAG  $92 \pm 4\%$ . Urinary RBP was not significantly different compared to  $T_0$  ( $P > 0.05$ ), with a mean difference of 37.92  $\mu\text{g/l}$  (95% CI: [-2.13; 77.97]).

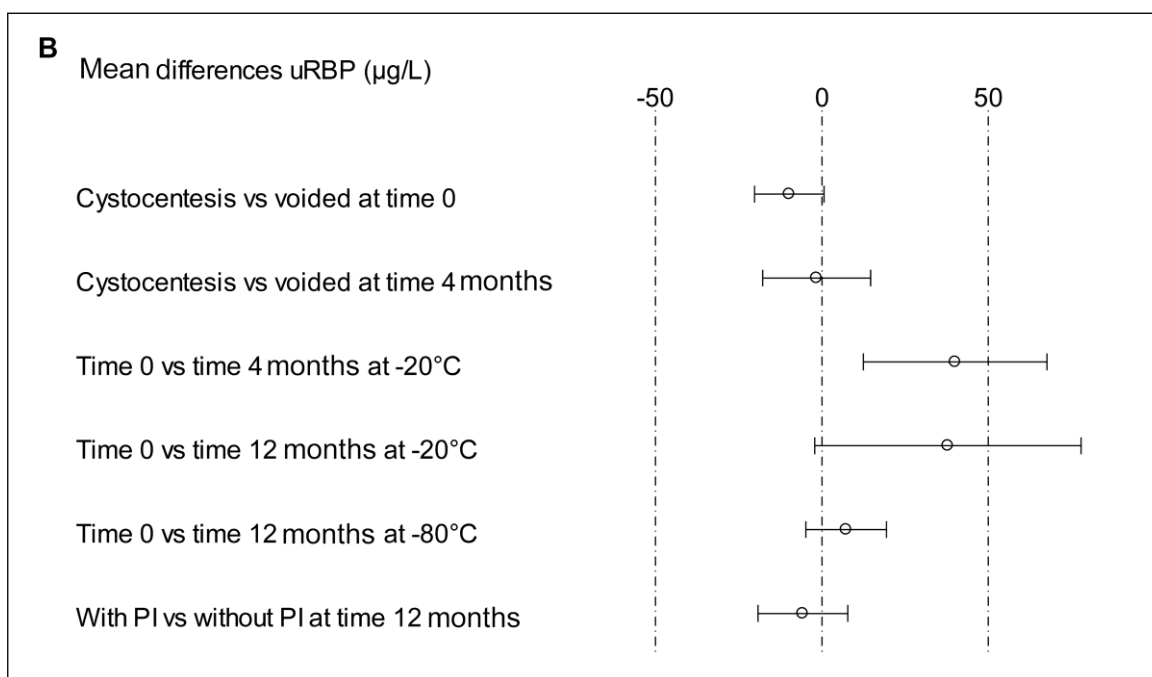
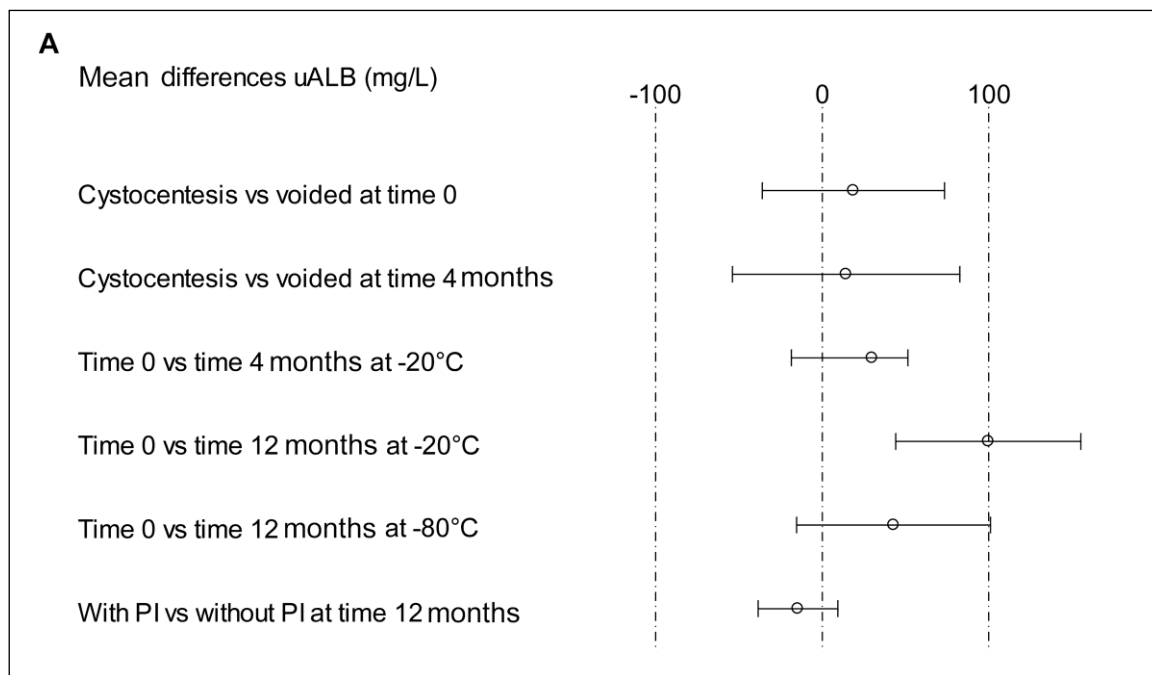
At  $T_{12 \text{ months}}$  after storage at  $-80^\circ\text{C}$ , only uNAG activity showed a significant decrease of 2.7 U/l (95% CI: [2.09; 3.3]) compared to  $T_0$  ( $P < 0.0001$ ), with a mean decrease of  $89 \pm 5\%$ . Urinary ALB and uRBP concentrations did not differ significantly from  $T_0$  ( $P > 0.05$ ), with a difference of 42.89 mg/l (95% CI: [-15.33; 101.1]) and 7.3  $\mu\text{g/l}$  (95% CI: [-4.84; 19.44]), respectively.

Samples with addition of PI were analyzed only at  $T_{12 \text{ months}}$  after storage at  $-80^\circ\text{C}$ . Urinary ALB, uRBP, and uNAG concentrations were not statistically different in samples with PI compared to samples stored without PI ( $P > 0.05$ ). Urinary NAG activity remained significantly lower compared to  $T_0$  ( $P < 0.0001$ ), regardless of PI addition.

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**Figure 1. Mean differences in urinary albumin (uALB; A), urinary retinol-binding protein (uRBP; B), and urinary N-acetyl-β-D-glucosaminidase (uNAG; C) concentrations between cystocentesis and voided samples in fresh urine and after storage for 4 months at -20°C, and between fresh urine samples and samples stored at -20°C and at -80°C for 4 and 12 months.**

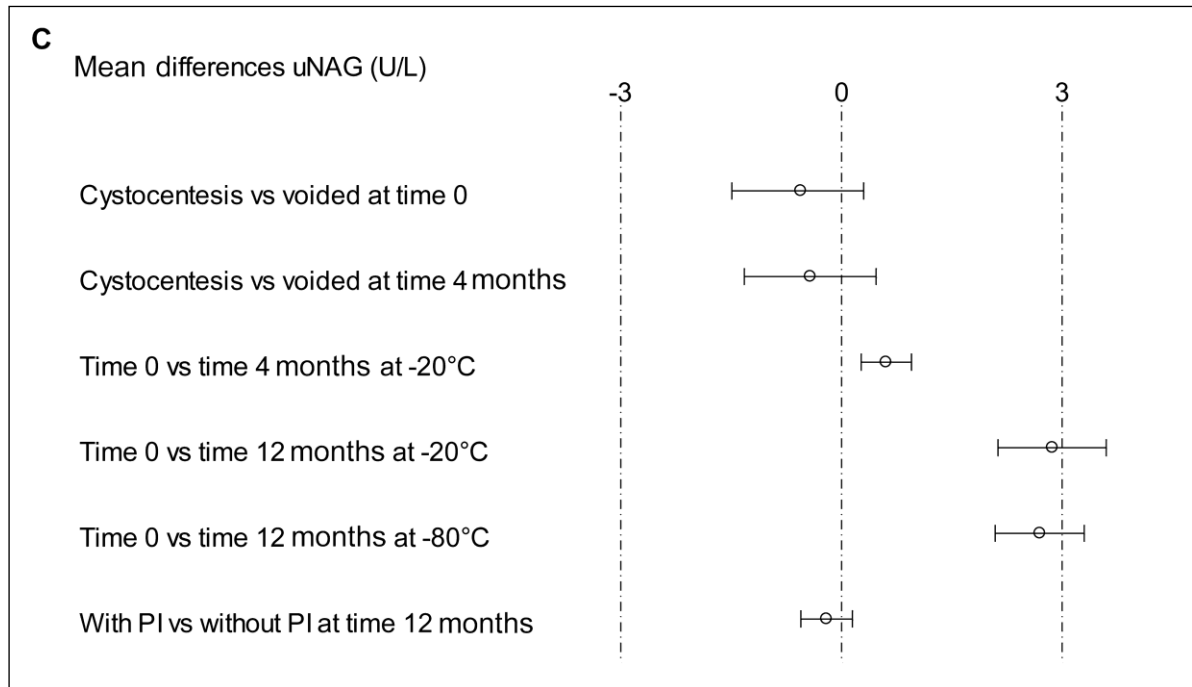
PI, protease inhibitor.





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Figure 1 (continued). Mean differences in urinary albumin (uALB; A), urinary retinol-binding protein (uRBP; B), and urinary N-acetyl- $\beta$ -D-glucosaminidase (uNAG; C) concentrations between cystocentesis and voided samples in fresh urine and after storage for 4 months at  $-20^{\circ}\text{C}$ , and between fresh urine samples and samples stored at  $-20^{\circ}\text{C}$  and at  $-80^{\circ}\text{C}$  for 4 and 12 months. PI, protease inhibitor.



As presented in Table 1, uALB, uRBP, and uNAG concentrations did not differ between blank samples and urine samples with 1+ hematuria, 1+ pyuria, and bacteriuria. Urinary ALB and uRBP, but not uNAG concentration, showed a very mild increase in samples with 3+ hematuria.

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**Table 1. Urinary marker concentrations in blank urine samples and urine samples with experimental hematuria, pyuria, and bacteriuria.**

uALB, urinary albumin; uRBP, urinary retinol-binding protein; uNAG, urinary N-acetyl- $\beta$ -D-glucosaminidase. BDL, below detection limit; RBC, red blood cells.

	Dog 1			Dog 2		
	uALB (mg/l)	uRBP ( $\mu$ g/l)	uNAG (U/l)	uALB (mg/l)	uRBP ( $\mu$ g/L)	uNAG (U/l)
Blank samples						
0h incubation	25.9	161.6	1.8	2.9	BDL	BDL
2h incubation	34.2	163.7	1.4	3.5	BDL	BDL
Hematuria						
RBC 1+	34.1	183.7	1.9	3.4	BDL	BDL
RBC 3+	40.5	217.1	1.9	8.1	58.3	BDL
Pyuria 1+	35.7	160.9	2.1	3.6	BDL	BDL
Bacteriuria	34.9	164.4	1.9	4.4	BDL	BDL

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#### **Discussion**

Results of the present study demonstrate that uALB, uRBP, and uNAG concentrations do not differ significantly between cystocentesis and voided urine samples. In contrast, storage conditions may affect uALB and uRBP concentrations and uNAG enzyme activity. Results also suggest that the addition of a PI cocktail does not preserve uNAG activity after long-term storage at  $-80^{\circ}\text{C}$ . Effects of experimental microscopic hematuria, pyuria, and bacteriuria on these urinary marker concentrations seem to be minimal, although these *in vitro* results do not reflect the complexity of an inflammatory process *in vivo*, and further research is needed.

In human medicine, there are many reports on urine sample storage conditions, their effect on protein concentrations and on subsequent data interpretation.<sup>14-18</sup> To the authors' knowledge, the effect of sampling method on urinary marker concentrations has never been reported in dogs or cats. In reports on uALB, uRBP, or uNAG in canine or feline urine, cystocentesis as well as catheterization and midstream voided urine have been used.<sup>9,19-20</sup> In contrast to voided samples, cystocentesis avoids contamination with lower urinary tract epithelial cells, proteases, and bacteria that may affect urinary markers.<sup>21</sup> When performing cystocentesis, care must be taken to avoid peripheral blood contamination leading to grossly discolored urine samples, since this may cause a falsely increased urinary albumin concentration.<sup>12</sup> However, results of the present study did not indicate a statistically significant difference in uALB, uRBP, or uNAG concentrations between cystocentesis and voided samples at  $T_0$  and at  $T_{4\text{ months}}$  after storage at  $-20^{\circ}\text{C}$ .

Despite this general finding, 1 dog with combined diabetes mellitus and Cushing's disease, and a negative urine culture, had increased uALB, uRBP, and uNAG concentrations in the voided sample (voided: uALB, 183.17 mg/l; uRBP, 71.1  $\mu\text{g/l}$ ; uNAG, 5.86 U/l; cystocentesis: uALB, 33 mg/l; uRBP, 29.8  $\mu\text{g/l}$ ; uNAG, 1.51 U/l). In this male castrated dog, macroscopic hematuria was not present in either the cystocentesis or voided sample. Sediment analysis of the cystocentesis sample did not reveal pyuria or inflammation. However, in the sediment of the voided sample, an increased amount of red blood cells, white blood cells, and epithelial cells were detected. Therefore, one hypothesis might be that urethral inflammation or preputial disease caused severe contamination of the urine sample, which affected the urinary markers. This finding suggests that other components of the inflammatory response, not mimicked by the *in vitro* experiment, may also play a role. Although, in general, a

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significant difference between cystocentesis and voided samples was not detected, possible interindividual variations should be considered.

To the authors' knowledge, there are no reports in the veterinary literature on stability of uRBP, uALB, and uNAG after frozen storage. At  $T_{4 \text{ months}}$  after storage at  $-20^{\circ}\text{C}$ , uALB concentration was comparable to  $T_0$ . In human medicine, there are contradictory reports on uALB stability. Results of the present study are in agreement with previous studies that describe no significant change in uALB concentration after storage at  $-20^{\circ}\text{C}$  for up to 6 months.<sup>22-23</sup> However, others detected a decrease of 20% in uALB concentration after 6 months at  $-20^{\circ}\text{C}$ .<sup>18</sup> In the present study, uRBP was significantly lower at  $T_{4 \text{ months}}$  compared to  $T_0$ . Nevertheless, this decrease was mild (40.11  $\mu\text{g/l}$ ), and the entire 95% CI was within a clinically acceptable range. Moreover, the RBP ELISA range of analytical variation for canine urine is 15.6% (mean interassay coefficient of variation + 2\*SD) and mean percentage decrease of uRBP reported herein was 15%.<sup>3</sup> Therefore, the decrease in uRBP may be due to assay variation. Urinary NAG was significantly lower at  $T_{4 \text{ months}}$  compared to  $T_0$ , with a mean percentage decrease of 20%. This finding corresponds to results of studies in human medicine, which demonstrated a 25.6% decrease in uNAG after 2 months of storage at  $-20^{\circ}\text{C}$ .<sup>24</sup>

While both uALB concentration and uNAG activity were significantly decreased at  $T_{12 \text{ months}}$  after storage at  $-20^{\circ}\text{C}$ , only uNAG was significantly lower compared to  $T_0$  after storage at  $-80^{\circ}\text{C}$ . For uALB, these findings are similar to previous results demonstrating a significant decline in human uALB after 12 months at  $-20^{\circ}\text{C}$ , but not at  $-80^{\circ}\text{C}$ .<sup>15</sup> In the present study, uNAG was dramatically decreased at  $T_{12 \text{ months}}$  with losses of 92% at  $-20^{\circ}\text{C}$  and of 89% at  $-80^{\circ}\text{C}$ . Addition of a PI cocktail had no effect on the preservation of uNAG. The decrease at  $-80^{\circ}\text{C}$  was unexpected because studies in human medicine indicated a largely preserved uNAG activity (84.6 %) after storage for 12 months at  $-70^{\circ}\text{C}$ .<sup>17</sup> Still, components of canine urine differ from human urine, and enzyme activities are influenced by many factors, such as temperature, pH, specific inhibitors, creatinine, urea, and electrolytes.<sup>25</sup> Alkaline pH ( $> 8$ ) causes a rapid decrease of uNAG, but urinary pH values in the present study ranged from 5.5 to 8, and only 1 sample had a pH of 8.<sup>25-26</sup>

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At T<sub>12 months</sub> after storage at -20°C, uRBP was not significantly lower compared to T<sub>0</sub>. This may seem contradictory because uRBP was mildly decreased at T<sub>4 months</sub>. However, the former decline is probably due to assay variability, and none of the observed differences is so large as to have clinical relevance. In the current study, storage at -20°C for 4–12 months seems to have only minimal effects on uRBP concentration. This finding is in contradiction with results obtained from human samples demonstrating decreased uRBP concentrations after storage at -20°C for 6–8 months. However, it must be noted that only a minority of samples in the latter study showed substantial underestimation.<sup>18</sup> At T<sub>12 months</sub> after storage at -80°C, the present study indicated no significant difference in uRBP compared to T<sub>0</sub>. This is in accordance with data from human medicine demonstrating no significant change in uRBP when stored at -70°C for 8 months.<sup>18</sup>

Guidelines advise diagnosticians to exclude urinary tract inflammation or infection (UTI) before identifying the origin of proteinuria because it influences UPC.<sup>27-28</sup> However, the effect on other urinary markers is currently unclear. In human medicine, there is no evidence that asymptomatic UTI causes proteinuria or albuminuria.<sup>29</sup> Results of the present pilot experiment suggest that experimental pyuria, 1+ and 2+ hematuria, and bacteriuria do not affect uALB, uNAG, and uRBP concentrations. However, these findings have to be interpreted with caution, because *in vitro* addition of leucocytes or bacteria to urine does not fully reflect an *in vivo* inflammatory process, with its complex interactions between stimulated leucocytes and bacteria involving inflammatory cytokines, bacterial products, and urinary molecules released from various sources. Samples with 3+ hematuria had a negligible increase in uALB and uRBP, probably caused by contamination of plasma albumin and RBP through addition of whole blood to the urine samples. These results are in agreement with previous data from canine urine samples showing no increased uALB in the majority of pyuric samples and a uALB above normal range only in macroscopically hematuric samples.<sup>12</sup> Two studies report similar canine uNAG activity in samples with or without hematuria, asymptomatic bacteriuria, or positive urine cultures.<sup>9,30</sup> One report showed increased uNAG concentration in dogs with pyelonephritis but not in dogs with lower UTI.<sup>31</sup> Effects of UTI on uRBP have not been reported in veterinary medicine. Data from human medicine, however, showed increased uRBP in patients with acute pyelonephritis, but not in patients with cystitis or asymptomatic bacteriuria.<sup>32</sup>

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Limitations of the present study are that data were obtained at only 3 time points (0, 4, and 12 months) in a relatively small sample group and that the effect of a PI was examined after 12 months at  $-80^{\circ}\text{C}$ , but not at  $-20^{\circ}\text{C}$ . The pilot experiment to assess effects of hematuria, pyuria, and bacteriuria is limited because it does not fully mimic the complexity of an *in vivo* cystitis. Therefore, further work is needed to evaluate the true influence of UTI on these urinary markers. The study was not designed to clarify underlying mechanisms of storage effects on the markers, but provides essential information regarding storage conditions for further research in the domain of urinary markers.

In conclusion, samples for analysis of uALB and uRBP can be stored at  $-20^{\circ}\text{C}$  for up to 4 months, whereas  $-80^{\circ}\text{C}$  is preferred for storage up to 12 months. Urinary NAG enzyme activity is less stable at  $-20^{\circ}\text{C}$  as well as  $-80^{\circ}\text{C}$ , with already substantial losses after 4 months at  $-20^{\circ}\text{C}$  and a more severe decline after 12 months at  $-80^{\circ}\text{C}$ .

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#### **Sources and manufacturers**

- a. Combur stick, Roche Diagnostics, Mannheim, Germany
- b. Calbiochem protease inhibitor cocktail set n° III, Merck KGaA, Darmstadt, Germany
- c. Canine albumin ELISA, Immunology Consultants Laboratory, Newberg, OR
- d. Retinol binding protein ELISA, Immundiagnostik AG, Bensheim, Germany
- e.  $\beta$ -N-Acetylglucosaminidase Assay Kit, Sigma-Aldrich, St. Louis, MO
- f. Multistix 8 SG, Bayer AG, Leverkusen, Germany
- g. Percoll, Sigma-Aldrich, St. Louis, MO
- h. Coulter Counter, Coulter Electronics Ltd., Buckinghamshire, England, United Kingdom
- i. Shandon Cytospin cytocentrifuge and Shandon Cytoclip slide clips, Thermo Scientific, Runcorn, Cheshire, United Kingdom
- j. Hemacolor, Merck KGaA, Darmstadt, Germany
- k. SAS version 9.1, SAS Institute Inc., Cary, NC

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### **CHAPTER III: *Sampling method and storage conditions for urinary markers***

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## **CHAPTER IV**

# **URINARY MARKERS IN HEALTHY DOGS AND IN DOGS WITH CHRONIC KIDNEY DISEASE**

**CHAPTER IV: *Urinary markers in healthy dogs and dogs with CKD***

**URINARY MARKERS IN HEALTHY YOUNG AND AGED DOGS AND IN DOGS  
WITH CHRONIC KIDNEY DISEASE**

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### **Summary**

Serum creatinine (screat) and urea concentrations only detect a decrease of > 75% of renal functional mass. Therefore, there is a need for markers that allow early detection and localization of renal damage. The objective was to investigate urinary albumin (uALB), C-reactive protein (uCRP), retinol binding protein (uRBP) and N-acetyl- $\beta$ -D-glucosaminidase (uNAG) concentrations in dogs with chronic kidney disease (CKD) compared with healthy controls and in healthy older dogs compared with young dogs. Ten dogs with CKD, 10 healthy young dogs (age 1-3 years) and 10 healthy older dogs (age > 7 years) without clinically relevant abnormalities on physical examination, hematology, biochemistry and urinalysis were included. Urinary markers were determined using an enzyme linked immunosorbent assay (ELISA) (uALB, uCRP and uRBP) or a colorimetric test (uNAG). Results were related to urinary creatinine (c). The fixed effects model or the Wilcoxon rank sum test were used to compare the different groups of dogs.

Urinary ALB/c, uRBP/c and uNAG/c were significantly higher in CKD dogs than in healthy dogs. No significant difference was found for uCRP, which was not detectable in the healthy dogs and only in 3 of the CKD dogs. Between the healthy young and older dogs, no significant difference was detected for any of the markers.

The urinary markers uALB/c, uRBP/c and uNAG/c were significantly increased in dogs with CKD compared to healthy controls. Additional studies are needed to evaluate the ability of these markers to detect renal disease before the onset of azotemia.

## **Introduction**

Chronic kidney disease (CKD) is an important cause of morbidity and mortality in dogs. The prevalence of CKD increases with age, with 15% of dogs over 10 years old being affected.<sup>1</sup> Early diagnosis may allow therapeutic intervention that prevents further damage and progressive decline of renal function. However, only a decrease of > 75% of renal functional mass will be detected by current diagnostic tests such as urea and serum creatinine (screat) concentrations.<sup>2</sup>

Unlike these insensitive serum tests, urinary markers are sensitive indicators of renal injury and also have the potential to reflect the site and severity of damage.<sup>3</sup> They include proteins categorized according to their molecular weight: high molecular weight (HMW), intermediate molecular weight (IMW) and low molecular weight (LMW) proteins.<sup>4</sup> In general, renal pathologic proteinuria may be due to increased glomerular filtration or tubular dysfunction causing impaired reabsorption of normally filtered protein.

Glomerular dysfunction leads to higher filtration of IMW proteins such as albumin and in more advanced stages to the presence of HMW proteins in the ultrafiltrate.<sup>4</sup> A urinary albumin concentration above normal, but below the limit of detection of conventional dipstick analysis (i.e. between 1 and 30 mg/dl) is defined as microalbuminuria. Several studies suggest that microalbuminuria may be an early indicator of glomerular disease in dogs.<sup>5-6</sup> An example of a HMW protein is C-reactive protein (CRP), an acute phase protein with an increased serum concentration in many inflammatory diseases.<sup>7</sup> To the authors' knowledge, urinary CRP (uCRP) has only recently been evaluated in 1 study, reporting an increased CRP-to-creatinine ratio (uCRP/c) in dogs with renal damage secondary to pyometra.<sup>8</sup>

Tubular dysfunction is reflected by urinary loss of LMW proteins or urinary enzymes.<sup>9</sup> Retinol binding protein (RBP) is a LMW protein associated with tubular impairment in humans and dogs with renal disease and in hyperthyroid cats.<sup>10-12</sup> Excessive amounts of urinary enzymes appear in urine due to leakage from damaged tubular cells.<sup>13</sup> One such enzyme is N-acetyl- $\beta$ -D-glucosaminidase (NAG), a lysosomal glycosidase present in proximal tubular cells. Urinary NAG has proven to be a useful tool in early detection of renal injury in various human diseases as reviewed by Skalova.<sup>14</sup> In veterinary medicine, an increased urinary NAG-to-creatinine ratio (uNAG/c) has been observed in dogs in various

#### **CHAPTER IV: *Urinary markers in healthy dogs and dogs with CKD***

stages of CKD<sup>15-16</sup>, leishmaniasis<sup>17</sup> and pyometra<sup>18</sup> and in dogs treated with nephrotoxic antibiotics<sup>19</sup>, non-steroidal anti-inflammatory drugs or glucocorticoids<sup>20</sup>.

Most studies in dogs have focused on 1 urinary marker, whereas reports on the combined measurement of glomerular and tubular markers in healthy and CKD dogs are scarce. Data about urinary marker assay validation likewise are scarce, especially for NAG.<sup>21</sup> Finally, little information exists on the urinary concentration of these markers in aging dogs.<sup>22</sup> Therefore, the aims of this study were first to validate a commercial canine albumin ELISA and NAG colorimetric test. Secondly, we wanted to compare 2 glomerular markers, urinary albumin (uALB) and uCRP, and 2 tubular markers, urinary RBP (uRBP) and NAG (uNAG) between healthy and CKD dogs. A third objective was to detect a possible age-related difference in these markers between healthy young and older dogs.

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### **Material and Methods**

#### ***Dogs***

The current study was performed at the Faculty of Veterinary Medicine, Ghent University, after approval by the Local Ethical Committee (approval number 2008/066). All owners agreed to participate in the study and signed an informed consent form.

In this cross-sectional study, 20 staff- and student-owned healthy dogs were recruited and divided into 2 groups according to their age: a group of young dogs (age 1-3 years) and a group of healthy older dogs ( $\geq 7$  years). Dogs were judged healthy based on history, physical examination, complete blood count, biochemistry profile and urinalysis (negative bacterial culture and urine protein-to-creatinine ratio, UPC  $< 0.5$ ). Dogs were excluded when medication possibly influencing renal function had recently been administered. Descriptive statistics for both groups of healthy dogs are presented in Table 1.

Ten privately owned dogs with CKD of all ages, breeds and both sexes were included. Diagnosis was based on clinical signs compatible with CKD (e.g., polyuria, polydipsia, weight loss, inappetence, vomiting), laboratory findings such as anemia, azotemia, electrolyte disturbances and urine specific gravity (USG)  $< 1.030$ . Dogs with CKD were classified according to the International Renal Interest Society (IRIS) into stages I to IV.<sup>23</sup> Exclusion criteria were the presence of concurrent infectious, neoplastic or endocrine diseases. Descriptive statistics for the CKD dogs are presented in Table 1.

Clinical signs were polyuria and polydipsia (8/10), lethargy (6/10), weight loss (5/10), decreased appetite or anorexia (5/10), vomiting (5/10) and diarrhea (2/10). Fecal examination of 1 dog with diarrhea indicated a *Giardia* and *Strongyloides stercoralis* infection. Two dogs had positive urine cultures (*Escherichia coli*). CKD dogs were IRIS stage I (n=2), III (n=2) or IV (n=6) and all were proteinuric (UPC  $> 0.5$ ). The 2 dogs in stage I had sreat concentrations of 1.39 mg/dl and 1.37 mg/dl and UPC ratios of 2.33 and 3.06, respectively. Ultrasound findings were hyperechoic renal cortices and a renal cortical cyst of 3 mm diameter in 1 dog and decreased kidney size (3.8 cm), hyperechoic parenchyma and decreased corticomedullary demarcation in the other dog. Juvenile renal disease was found as underlying cause for CKD in 4 dogs: a Leonberger, a Boxer, a Bull Mastiff and a Schapendoes (ages 2.3, 0.7, 2.6 and 0.5 years, respectively). The Boxer had a littermate that had been euthanized because of end-stage renal disease and the Schapendoes was diagnosed with polycystic kidney disease.

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**Table 1. Group descriptive statistics (median, range) for the healthy and CKD dogs.**

\*\* indicates a significant difference ( $p < 0.0001$ ); M, male intact; F, female intact; FN, female neutered; MN, male neutered; screat, serum creatinine; UPC, urine protein-to-creatinine ratio; USG, urine specific gravity.

Variable	Healthy dogs			CKD dogs
	Young dogs	Older dogs	Total	
number of dogs	10	10	20	10
age (years)	2.4 (1.1-2.9)**	8.3 (7-10.9)**	5 (1.1-10.9)	4.7 (0.5-10.5)
gender	2 M, 5 F, 1 MN, 2 FN	2 M, 4 F, 2 MN, 2 FN	4 M, 9 F, 3 MN, 4 FN	2 M, 4 F, 2 MN, 2 FN
bodyweight (kg)	23.8 (8.8-36.0)	24.6 (8.7-39)	24.5 (8.7-39)	17.4 (4.2-40.1)
screat (mg/dl)	0.99 (0.69-1.24)	0.92 (0.42-1.10)	0.94 (0.42-1.24)**	5.51 (1.37-15.94)**
urea (mg/dl)	33.99 (26.01-43.00)	26.13 (18.98-43.00)	29.52 (18.98-43.00)**	362.52 (95.98-689.01)**
UPC	0.08 (0.06-0.11)	0.1 (0.06-0.47)	0.08 (0.06-0.47)**	1.9 (0.63-3.39)**
USG	1.040 (1.008-1.050)	1.030 (1.016-1.049)	1.035 (1.008-1.050)**	1.010 (1.008-1.017)**

Concurrent heart disease was present in 1 dog with aortic and pulmonary valve stenosis (Cairn Terrier) and in 2 dogs with mitral valve disease, International Small Animal Cardiac Health Council (ISACHC) class Ia (Maltese) and class II (Cavalier King Charles). Nine dogs were newly diagnosed and referred to our clinic for further diagnostic work-up. In 1 dog, the diagnosis of CKD had been made 13 months earlier but the owner had stopped feeding the renal diet and stopped the ACE inhibitor therapy for several months. Six dogs had not yet received any treatment and 4 dogs had already been treated with anti-emetics, gastroprokinetics, antibiotics or fluid therapy by their referring veterinarians. The dog with ISACHC class II mitral valve disease was the only dog on an ACE inhibitor (benazepril) for 12 months.



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### ***Sample collection***

Morning urine samples were collected by cystocentesis (10 ml, 22 gauge needle). Urinalysis consisting of dipstick analysis<sup>a</sup>, USG, UPC, sediment analysis and bacterial culture, was performed. After centrifugation (3 min at 447 x g), the supernatant was divided into aliquots of 0.5 ml and stored at -80°C until analysis of each marker after thawing on ice.

### ***Laboratory methods***

Because spot urine samples were used, urinary concentration of each biomarker was related to urinary creatinine (c) and expressed as a ratio.<sup>19</sup> Urinary creatinine concentration was measured by the modified Jaffé reaction using picric acid.<sup>24</sup>

### ***Validation of the albumin ELISA and NAG colorimetric assay***

As an indication of assay precision, within-day coefficient of variation (CV) was calculated from 30 duplicate samples assayed on the same day and between-day CV from 15 samples assayed on separate days, respectively. In addition, between-day variation was assessed using Lin's sample concordance correlation coefficient (Lin's CCC,  $\rho_c$ ), which indicates the degree of deviation from total agreement between 2 repetitive measurements of the same sample.<sup>25</sup>

Accuracy of the assays was assessed by evaluation of linearity under serial dilution.<sup>26</sup> Urinary concentration in diluted samples was plotted against dilution factor and linear regression analysis was performed.

Assay sensitivity was determined using the mean and standard deviation (SD) of blank samples to define the lowest concentration of albumin and NAG that can be reliably distinguished from a blank measurement. The limit of detection (LOD) was calculated as mean blank result + 2.6\*SD.<sup>27</sup>

### ***ALB, CRP and RBP ELISA***

UALB and uCRP concentration were measured with commercial canine specific sandwich ELISA kits<sup>b</sup> and uRBP with a human ELISA kit<sup>b</sup>, previously validated in our laboratory for use with canine urine.<sup>28</sup> A microtiter plate precoated with affinity-purified antibodies for canine albumin, CRP or human RBP was used. Wells were filled with 100  $\mu$ l of diluted urine samples or standards (albumin, 12.5-400 ng/ml; CRP, 3.125-200 ng/ml; RBP, 7.8-250 ng/ml) and incubated. After several wash steps, peroxidase-labeled albumin, CRP or RBP antibody conjugate was added. After incubation and wash steps, each well was filled

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with 100 µl of tetramethylbenzidin substrate and incubated. Addition of sulfuric acid stopped the colorimetric reaction and absorbance was measured using an ELISA plate reader<sup>c</sup> at a wavelength of 450 nm with 650 nm as a reference. A 4 parameter logistic curve fitting program<sup>d</sup> was used to generate the standard curve and calculate albumin, CRP or RBP concentrations in the urine samples.

### *NAG colorimetric activity assay*

Urinary NAG activity was calculated with a colorimetric assay<sup>e</sup>. The NAG enzyme hydrolyzes the 4-nitrophenyl-N-acetyl-β-D-glucosaminide substrate and releases p-nitrophenol (PNP). Addition of the basic stop solution causes ionization of the PNP to the p-nitrophenolate ion. The absorbance of the latter ion was measured at 405 nm with a plate reader<sup>c</sup>. NAG activity was calculated using a standard formula and divided by urinary creatinine concentration to determine the uNAG/c (U/g) = NAG activity (U/l) / urinary creatinine (g/l).<sup>15,19</sup>

### *Statistical analysis*

Analyses were performed with a commercial software program<sup>f</sup>. For normally distributed data, the linear model with normally distributed random error term and dog group (healthy and CKD) and age category (young and older) as categorical fixed effects was fitted. An *F*-test was used to compare the 2 dog groups. When the normal distribution assumption did not hold, the Wilcoxon rank sum test stratified for age category was performed to compare the CKD dogs with the healthy dogs.

In a 2nd analysis, the young healthy dogs were compared with the old healthy dogs using the same analysis techniques as above, but only for the healthy dogs and without including age category as an adjusting covariate.

A global significance level of 5% was used to test, leading to a Bonferroni-adjusted significance level of 2.5% for each of the 2 comparisons above.

Correlations between different urinary markers and between the markers and other variables (screat, urea, UPC and IRIS classification) were determined using Pearson's correlation coefficient for normally distributed data or Kendall's τ for the other data. Results are expressed as median (range).

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### Results

#### *Validation of the albumin and NAG assays*

Precision and sensitivity of the albumin ELISA and NAG colorimetric assay are presented in Table 2. Both assays had satisfactory variability with within-day CV < 10% and a between-day CV < 15%. Moreover, a  $\rho_c > 0.95$  indicated substantial agreement between repetitive measurements of samples on separate days. Regression analysis after serial dilution of urine samples showed a linear relationship between albumin concentration or NAG activity and dilution factor (Figure 1).

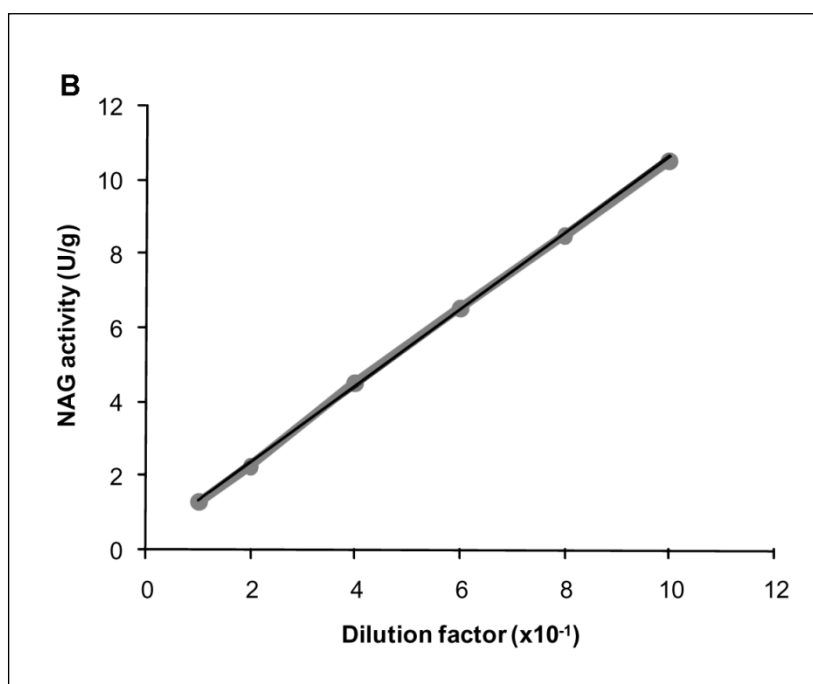
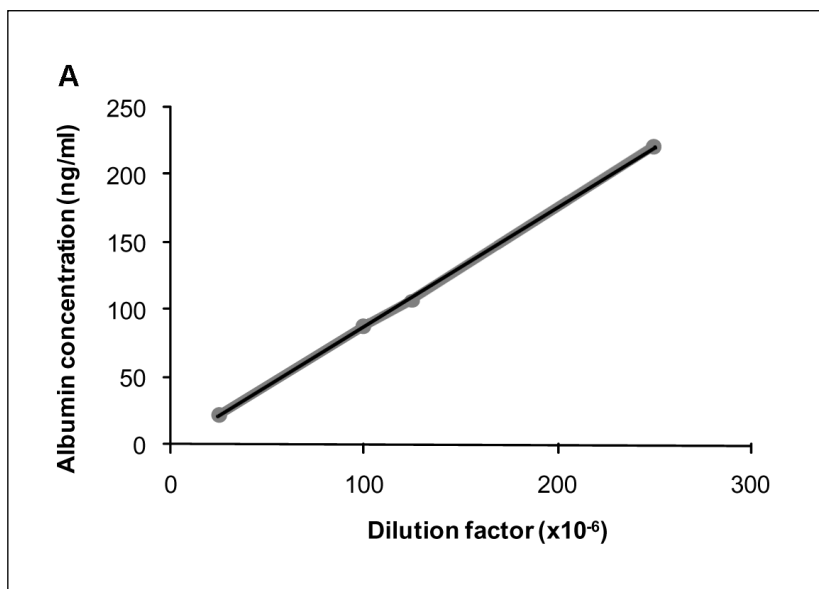
**Table 2. Assay characteristics for canine albumin ELISA and NAG colorimetric assay.**

ALB, albumin ELISA; NAG, N-acetyl- $\beta$ -D-glucosaminidase colorimetric assay; LOD, limit of detection; CV, coefficient of variation;  $\rho_c$ , Lin's concordance correlation coefficient.

Assay characteristic	ALB	NAG
Sensitivity : LOD	15.1 ng/ml	0.84 U/l
Precision: Within-day CV (%)	5.2	4.9
Between-day CV (%)	12.0	8.1
$\rho_c$	0.98	0.98

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**Figure 1. Sequential dilution of a urine sample from a dog with CKD.** (A) urinary albumin concentration (ng/ml) plotted against dilution factor ( $\times 10^{-6}$ ). The linear regression equation was  $y = 0.8812x - 0.3507$  ( $r^2 = 0.9995$ ). (B) urinary NAG activity (U/l) plotted against dilution factor ( $\times 10^{-1}$ ). The linear regression equation was  $y = 1.0296x + 0.3107$  ( $r^2 = 0.995$ ).  
CKD, chronic kidney disease; NAG, N-acetyl- $\beta$ -glucosaminidase.



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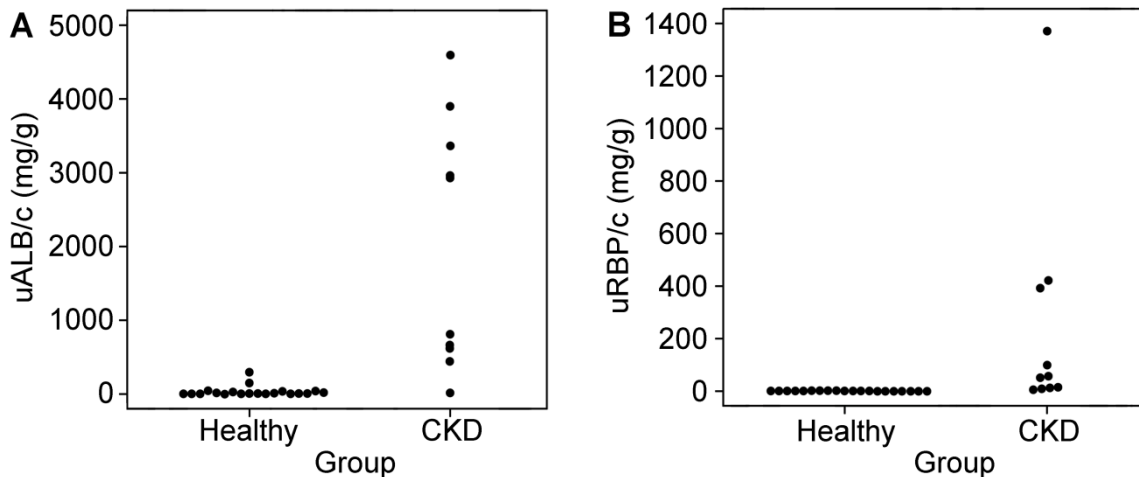
### *Urinary markers*

Results for uALB/c, uRBP/c and uNAG/c in healthy and CKD dogs are presented in Figure 2 and Table A1 and A2. There was a highly significant difference between healthy and CKD dogs for uALB/c, uRBP/c and uNAG/c ( $p < 0.001$ ), but not for uCRP ( $p = 0.07$ ). In the healthy dogs, median uALB/c was 7.3 mg/g (1.5-296.5) and in the dogs with CKD uALB/c was 1868.6 (12.7-4594.6). None of the healthy dogs had any detectable uCRP, whereas 3 of the CKD dogs did, with uCRP/c of 84.3  $\mu\text{g/g}$ , 179.8  $\mu\text{g/g}$  and 236.9  $\mu\text{g/g}$ . uRBP/c was 0.10 mg/g (0-0.89) in the healthy dogs whereas dogs with CKD had a median ratio of 53.4 (6.8-1372.2). The healthy dogs had a median uNAG/c of 2.2 U/g (1.2-5.5) and the CKD dogs had a uNAG/c of 5.7 U/g (1.5-9.5).

When comparing the healthy young with the healthy older dogs, no significant differences were found for uALB/c, uCRP/c, uRBP/c or uNAG/c ( $p > 0.025$ ). uALB/c in the young dogs was 4.7 mg/g (1.5-46.3) and in the older dogs 17.8 (3.3-296.5). In the young dogs, median uRBP/c was 0.1 mg/g (0-0.2) and in the older dogs also 0.1 (0-0.9). Median uNAG/c was 2.5 U/g (1.6-5.5) in the young dogs and in the older dogs it was 2 (1.2-5.2).

**Figure 2. Scatter plot of uALB/c (mg/g) (A) and uRBP/c (mg/g) (B) in healthy and CKD dogs.** All three markers were significantly different ( $p < 0.001$ ) between groups.

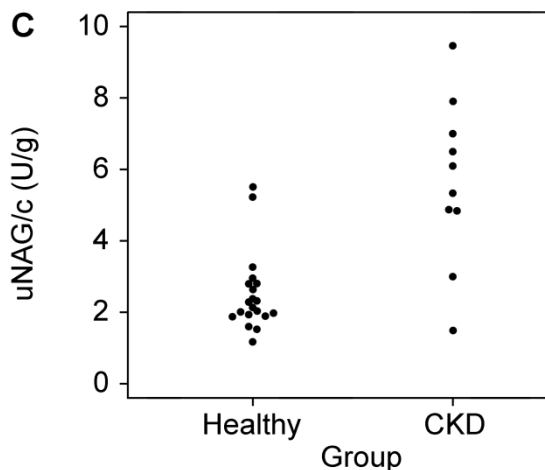
uALB/c, urinary albumin-to-creatinine ratio; uRBP/c: urinary retinol-binding protein-to-creatinine ratio; CKD, chronic kidney disease.



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**Figure 2 (continued).** Scatter plot of uNAG/c (U/g) (C) in healthy and CKD dogs. This marker was also significantly different ( $p < 0.001$ ) between both groups.

uNAG/c, urinary N-acetyl- $\beta$ -glucosaminidase-to-creatinine ratio; CKD, chronic kidney disease.



Correlations between different urinary markers and between the markers and other variables are shown in Table 3. The highest correlations were found between uALB/c and UPC ( $r = 0.96$ ,  $p < 0.001$ ), uALB/c and uRBP/c ( $r = 0.65$ ,  $p < 0.001$ ), uRBP/c and UPC ( $r = 0.74$ ,  $p < 0.001$ ) and uRBP/c and IRIS stage ( $r = 0.72$ ,  $p < 0.001$ ). uRBP/c also showed moderate correlations to screat ( $r = 0.51$ ,  $p < 0.001$ ) and urea ( $r = 0.56$ ,  $p < 0.001$ ), and uALB/c to IRIS stage ( $r=0.53$ ,  $p < 0.025$ ), but not to screat and urea. uCRP/c was weakly to moderately associated with screat ( $r = 0.38$ ,  $p < 0.025$ ), IRIS stage ( $r=0.52$ ,  $p < 0.025$ ) and urea ( $r=0.41$ ,  $p < 0.025$ ), but not with UPC ( $p > 0.025$ ). uNAG/c was moderately correlated to IRIS stage ( $r=0.49$ ,  $p < 0.025$ ), urea ( $r = 0.42$ ,  $p < 0.025$ ) and UPC ( $r=0.51$ ,  $p < 0.025$ ), but not to screat.

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**Table 3. Correlations (r) for the urinary markers, screat, IRIS stage, urea and UPC.**

\* indicates a significant correlation ( $p < 0.025$ ); \*\* indicates a significant correlation ( $p < 0.001$ ); screat, serum creatinine; IRIS, International Renal Interest Society; UPC, urine protein-to-creatinine ratio; uALB/c, urinary albumin-to-creatinine ratio; uCRP/c, urinary CRP-to-creatinine ratio; uRBP/c, urinary retinol binding protein-to-creatinine ratio; uNAG/c, urinary N-acetyl- $\beta$ -D-glucosaminidase-to-creatinine ratio.

	<b>screat</b>	<b>IRIS stage</b>	<b>UREA</b>	<b>UPC</b>	<b>uALB/c</b>	<b>uCRP/c</b>	<b>uRBP/c</b>	<b>uNAG/c</b>
<b>uALB/c</b>	r=0.27	r=0.53*	r=0.35	r=0.96**	r=1	r=0.27	r=0.65**	r=0.56*
<b>uCRP/c</b>	r=0.38*	r=0.52*	r=0.41*	r=0.32	r=0.27	r=1	r=0.43*	r=-0.02
<b>uRBP/c</b>	r=0.51**	r=0.72**	r=0.56**	r=0.74**	r=0.65**	r=0.43*	r=1	r=0.49*
<b>uNAG/c</b>	r=0.33	r=0.49*	r=0.42*	r=0.51*	r=0.56*	r=-0.02	r=0.49*	r=1

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### **Discussion**

Results of the present study indicate satisfactory assay characteristics of the quantitative canine albumin ELISA and NAG colorimetric assay when applied for evaluation of urine. Furthermore, uALB, uCRP, uRBP concentrations and uNAG activity were found to be significantly higher in dogs with CKD compared to healthy controls. No age-related difference in urinary markers was detected between young and older healthy dogs.

The assays used in this study were able to measure canine uALB and uNAG in a linear manner with within- and between-run imprecision of 5.2 and 12%, and 4.9 and 8.1%, respectively. As a comparison, for uALB ELISA's, other studies have reported within- and between-run imprecision ranging between 2 to 17% and 4.5 to 21.7%, respectively.<sup>29-30</sup> For the NAG colorimetric assay, within- and between-run CV's of 2.8% and 11.9% have been reported.<sup>31</sup> In research settings, where samples frequently are analyzed in the same run, these levels of imprecision are considered acceptable, but for diagnostic purposes steps to decrease between-run variability should be taken.

In the current study, both quantitative as well as the qualitative aspects of proteinuria were evaluated by measuring several proteins as candidate urinary markers for glomerular and tubular damage. Because albuminuria is the earliest detectable form of proteinuria and all dogs with CKD had a UPC > 0.5, the strong correlation between uALB/c and UPC was expected.<sup>32</sup> This strong correlation also was found in a study in cats with CKD, in which UPC also was predictive for survival.<sup>30</sup>

The use of microalbuminuria as a marker of early CKD is supported by several studies in dogs predisposed to glomerular disease because of a genetic cause or a heartworm infection.<sup>5-6</sup> In these dogs, microalbuminuria reflected the onset of disease more rapidly than the UPC. However, in the present study, most of the dogs had advanced renal disease and it was not the objective to prospectively evaluate uALB/c as an early marker of CKD.

This study is the first to describe uCRP concentrations in dogs with CKD. To the authors' knowledge, increased uCRP/c ratios only have been described in dogs with pyometra, and uCRP has never been evaluated in humans.<sup>8</sup> uCRP was not detected in the healthy dogs and increased uCRP/c ratios were present in 3 out of 10 CKD dogs. No increase in uCRP/c was detected in the 2 dogs with positive urine cultures or in the dog with a positive fecal examination. Although no statistically significant difference could be detected between



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CKD and control dogs, the presence of uCRP in dogs with CKD still is an interesting finding. For CRP to appear in urine, its plasma concentration must be increased and the glomerular barrier must be sufficiently damaged to allow HMW protein filtration. Thus, 1 possible hypothesis is that the increased uCRP/c in these 3 CKD dogs reflects an inflammatory response leading to increased plasma concentrations and subsequent leakage of CRP through the damaged glomerular barrier.

In humans, inflammation and oxidative stress start early in the process of failing kidney function and mild increases in CRP concentration are present even in patients with moderate renal impairment.<sup>33-34</sup> Among other causes, decreased renal clearance of CRP, pro-inflammatory cytokines such as interleukin-6 or both, and uremia are suggested reasons for chronic inflammation in these human patients.<sup>35</sup> This might also have been the cause in 1 dog with increased uCRP/c, which was in an advanced staged of CKD (IRIS stage IV) with severe clinical signs (e.g. vomiting, hemorrhagic diarrhea). In humans, serum CRP concentration also is a predictor of cardiovascular disease, such as congestive heart failure.<sup>36</sup> Interestingly, 2 of the dogs with increased uCRP/c were older dogs that also had mitral valve disease. One dog with aortic and pulmonary valve stenosis had a normal uCRP/c. To obtain better insight into the role of inflammation in canine CKD, plasma and urinary concentrations of CRP and other pro-inflammatory mediators should be evaluated in a larger number of dogs.

URBP was measured as a 1st marker of tubular dysfunction. In most mammals, this LMW protein circulates in plasma complexed with a second protein (transthyretin) and binds vitamin A, which prevents RBP excretion. However, dogs have high concentrations of transthyretin-uncomplexed RBP that is filtered by the glomeruli. Under physiologic conditions, the filtered RBP is almost completely reabsorbed by megalin-mediated endocytosis in the proximal tubular cells, but tubular dysfunction leads to excessive amounts of uRBP.<sup>10,37</sup> In our study, uRBP/c was significantly higher in dogs with CKD compared to healthy controls and highly correlated with screat, urea, UPC and IRIS-stage, which corroborates results from previous studies documenting increased uRBP/c ratios in CKD dogs.<sup>10,38</sup>

As a second marker for tubular dysfunction, uNAG was determined. Indeed, in humans increased uNAG/c in renal disease does not originate from filtration due to glomerular damage but from tubular epithelial cells.<sup>39-40</sup> This enzyme was found to be

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significantly higher in the CKD than in the healthy dogs, although there was large overlap in uNAG/c ratios between the 2 groups. In the present study, uNAG/c in CKD dogs (median, 5.5 U/g; range, 1.5-9.5 U/g) was lower than observed in 2 previous reports in 9 CKD dogs (mean  $\pm$  SD, 17.1  $\pm$  7.9 U/g) and 7 CKD dogs (median, 25.4 U/g; range, 15.7-136.8 U/g), respectively.<sup>15-16</sup> Possible explanations for this difference include variation in laboratory techniques for NAG analysis and dog-related factors, such as stage of CKD. Enzymuria may be a marker of active disease damaging the renal cells. When the primary cause of the damage has disappeared, enzymuria might become minimal despite pronounced structural damage and permanent loss of tubular cells.<sup>40</sup> To further investigate this hypothesis, additional studies are needed to determine uNAG/c in dogs with acute renal failure as well as in dogs with various stages of CKD.

Urinary tract infection (UTI) was an exclusion criterion for the healthy dogs, but 2 of the CKD dogs had positive urine cultures. The effect of lower UTI on microalbuminuria and uNAG/c seems to be minimal in dogs, but an increased uNAG/c ratio is reported in the presence of pyelonephritis.<sup>15,41</sup> In these 2 dogs, no ultrasonographic signs of pyelonephritis were detected. To the authors' knowledge, the effect of UTI on uRBP and uCRP is unknown in dogs. In humans, microalbuminuria and uCRP, uRBP and uNAG concentrations are increased in patients with upper UTI, but not in patients with cystitis or asymptomatic bacteriuria.<sup>42-45</sup>

The degree of correlation between the urinary markers and routine variables such as screat, urea and UPC differed for each urinary marker. This is not unexpected, because these markers might be more sensitive indicators of renal damage and some may reflect renal dysfunction at another site rather than serving as an indirect glomerular marker as does screat. Different markers each may reflect different pathophysiologic processes. UALB/c was not correlated with screat and urea whereas uRBP/c was strongly correlated with both. Progression of CKD in humans and dogs, respectively, may be initiated by glomerular changes subsequently leading to interstitial injury.<sup>4,46-47</sup> It is mainly the tubulointerstitial inflammatory process that precedes renal scarring and is associated with a decreased renal function. In this more advanced stage, large amounts of LMW proteins such as RBP appear in urine. Negatively-charged IMW proteins such as albumin are a sign of mildly altered glomerular permeability and already appear in urine in an early stage. Although uCRP/c was moderately to weakly correlated to screat, urea and UPC, this finding needs to be interpreted

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with caution because only 3 dogs had positive results. As in the present study, a report on urinary enzymes in humans with glomerulonephritis also demonstrated a correlation between uNAG/c and proteinuria and the absence of an association between uNAG/c and screat.<sup>48</sup> The current hypothesis is that urinary enzymes correlate better with tubulointerstitial damage than with an indirect measurement of glomerular filtration rate such as screat.

In the healthy dogs, a median uALB/c of 7.3 mg/g (range, 1.5-296.5) was measured. Cut-off values for uALB/c ratios remain to be properly established in a large number of healthy dogs. Usually, a normal range is calculated as mean uALB/c + 2\*SD of the healthy control group.<sup>49</sup> In 2 previous reports, uALB/c either ranged from 5 to 82 mg/g (n=6) or mean uALB/c  $\pm$  SD was 40  $\pm$  80 mg/g (n=10).<sup>49-50</sup> Three of 20 healthy dogs in the current study had uALB/c ratios above the upper limit of 42 mg/g (mean + 2\*SD of the healthy young dogs). Interestingly, 2 of these 3 dogs were older dogs with uALB/c ratios well above the cut-off (152.6 and 296.5 mg/g) and another 2 older dogs had uALB/c ratios near the cut-off (40.4 mg/g and 41.5 mg/g). Previous data indicated a higher prevalence of microalbuminuria with increasing age.<sup>22</sup> This finding might be related to the higher prevalence of glomerular lesions and CKD in aging dogs.<sup>1,51</sup> However, in the present study no renal biopsies were taken due to ethical considerations. As for the CKD dogs, knowledge of the histopathologic lesions would unlikely have therapeutic or prognostic implications in advanced disease stages.

UNAG/c ratios in the healthy dogs (median, 2.2 U/g; range, 1.2-5.5 U/g) were in agreement with previous reports.<sup>15,20,52</sup> The present study is the first to report uNAG/c ratios in older healthy dogs. No significant difference was found between the young and older healthy dogs. Nevertheless, the true effect of age is best assessed in a longitudinal follow-up of the same individuals. In humans, uNAG is higher in children than in adults.<sup>53</sup> This is mainly due to changes in muscle mass and consequently in creatinine excretion.<sup>54</sup> Therefore, in human medicine each laboratory establishes its own reference ranges for different age categories and patients then are compared against age-matched controls.<sup>53,55</sup>

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In conclusion, combined use of glomerular and tubular markers in conjunction with traditional tests may provide more detailed information about the extent and location of renal damage. The urinary markers discussed in this study appear to be promising as non-invasive tools for the diagnosis of CKD in dogs. Age-related differences seem to be minimal between young adult and older healthy dogs. These results should encourage in-depth investigation of urinary markers in larger numbers of dogs classified according to their etiology and stage of renal disease.

### **Sources and manufacturers**

- a. Combur stick, Roche Diagnostics, Germany
- b. Immunology Consultants Laboratory, Newberg, USA
- c. Multiskan MS<sup>®</sup>, LabSystems Thermo Fisher Scientific, Waltham, USA
- d. Deltasoft JV<sup>®</sup>, BioMetallics Incorporated, Princeton, USA
- e. Sigma-Aldrich<sup>®</sup>, St-Louis, Missouri
- f. SAS version 9.1, SAS Institute. Inc., Cary, IN

**CHAPTER IV: Urinary markers in healthy dogs and dogs with CKD**

**Addendum**

**Table A1. Individual data of the healthy dogs.** Dogs 1-10 belong to the group of young dogs and dogs 11-20 to the group of older dogs.

screat, serum creatinine; UPC, urinary protein-to-creatinine ratio; uRBP/c, urinary retinol-binding protein to-creatinine ratio; uNAG/c, urinary N-acetyl- $\beta$ -D-glucosaminidase-to-creatinine ratio; uALB/c, urinary albumin-to-creatinine ratio; uCRP/c, urinary C-reactive protein-to-creatinine ratio; BDL, below detection limit.

Healthy dogs	Age (years)	screat (mg/dl)	ureum (mg/dl)	UPC	uRBP/c (mg/g)	uNAG/c (U/g)	uALB/c (mg/g)	uCRP/c ( $\mu$ g/g)
Dog 1	2.9	0.90	29.01	0.08	0.10	2.7	2.8	BDL
Dog 2	1.5	0.89	26.01	0.08	0.07	2.8	1.5	BDL
Dog 3	2.9	1.02	30.99	0.07	0.14	3.0	5.7	BDL
Dog 4	1.1	0.82	32.97	0.07	0.12	5.5	46.3	BDL
Dog 5	1.7	0.69	43.00	0.11	0.14	3.3	15.7	BDL
Dog 6	2.9	1.24	37.00	0.06	BDL	2.1	1.7	BDL
Dog 7	1.9	1.14	41.98	0.11	0.19	2.3	31.7	BDL
Dog 8	2.9	0.95	35.02	0.07	BDL	1.6	3.1	BDL
Dog 9	2.9	1.11	41.02	0.08	BDL	1.9	3.7	BDL
Dog 10	1.25	1.15	27.03	0.08	0.14	2.0	8.6	BDL
Dog 11	7.2	0.89	30.03	0.07	BDL	1.2	3.3	BDL
Dog 12	8.3	0.87	18.98	0.07	0.04	1.9	13.2	BDL
Dog 13	8.1	1.01	25.23	0.17	0.89	2.8	152.6	BDL
Dog 14	10.8	0.93	41.02	0.12	0.13	1.5	40.4	BDL
Dog 15	10	0.95	27.03	0.06	BDL	2.0	5.1	BDL
Dog 16	10.9	0.76	21.98	0.07	BDL	1.9	6.0	BDL
Dog 17	8.3	0.98	21.98	0.08	BDL	2.0	5.0	BDL
Dog 18	8.7	1.09	43.00	0.19	0.23	5.2	41.5	BDL
Dog 19	7.5	0.90	19.22	0.11	0.43	2.3	22.5	BDL
Dog 20	7	0.42	27.03	0.47	0.44	2.4	296.5	BDL

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**Table A2. Individual data of the dogs with CKD.**

CKD, chronic kidney disease, screat, serum creatinine; UPC, urinary protein-to-creatinine ratio; uRBP/c, urinary retinol-binding protein to-creatinine ratio; uNAG/c, urinary N-acetyl- $\beta$ -D-glucosaminidase-to-creatinine ratio; uALB/c, urinary albumin-to-creatinine ratio; uCRP/c, urinary C-reactive protein-to-creatinine ratio; BDL, below detection limit.

<b>Dogs with CKD</b>	<b>Age (years)</b>	<b>screat (mg/dl)</b>	<b>ureum (mg/dl)</b>	<b>UPC</b>	<b>uRBP/c (mg/g)</b>	<b>uNAG/c (U/g)</b>	<b>uALB/c (mg/g)</b>	<b>uCRP/c (<math>\mu</math>g/g)</b>	<b>IRIS Stage</b>
Dog 1	2.6	15.94	689.01	0.90	392.88	4.9	444.9	179.8	IV
Dog 2	0.9	4.64	370.03	0.63	56.62	9.5	624.2	BDL	III
Dog 3	10.5	1.41	190.99	2.33	11.80	7.0	2959.2	BDL	I
Dog 4	2.3	5.66	215.02	3.39	50.24	5.3	3900.0	BDL	IV
Dog 5	9.5	1.37	95.98	3.06	6.77	7.9	4594.6	BDL	I
Dog 6	10.25	5.35	447.45	3.34	421.92	1.5	2924.9	236.9	IV
Dog 7	0.7	2.31	140.00	0.92	13.78	6.1	812.4	BDL	III
Dog 8	6.7	7.40	483.00	1.46	1372.18	4.8	658.3	84.3	IV
Dog 9	7.4	7.18	355.02	2.67	98.61	6.5	3364.5	BDL	IV
Dog 10	0.5	7.73	455.98	0.97	9.25	3.0	12.7	BDL	IV

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**CHAPTER V**  
**RENAL FUNCTION IN DOGS WITH CUSHING'S**  
**SYNDROME**

**CHAPTER V: § 5.1**

**§ 5.1 HYPERCORTISOLISM AFFECTS GLOMERULAR AND TUBULAR  
FUNCTION IN DOGS**

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**Summary**

## CHAPTER V: § 5.1

Renal function was assessed in 25 dogs with Cushing's syndrome and in 12 healthy controls. Routine renal parameters and glomerular filtration rate (GFR) were measured and urinary biomarkers such as urinary albumin (uALB), urinary immunoglobulin G (uIgG), and urinary retinol-binding protein (uRBP) were assessed by ELISA. Urinary N-acetyl- $\beta$ -D-glucosaminidase (uNAG) was determined colorimetrically. All urinary markers were indexed to urinary creatinine concentration (c). Plasma exo- ( $Cl_{\text{exo}}$ ) and endo-iohexol ( $Cl_{\text{endo}}$ ) clearance were used to measure GFR.

Based on a Mann-Whitney *U*-test, urea and  $Cl_{\text{exo}}$  did not differ, serum creatinine (screat) was significantly lower, and urinary protein-to-creatinine ratio (UPC), uALB/c, uIgG/c, uRBP/c, uNAG/c and  $Cl_{\text{endo}}$  were higher in the dogs with Cushing's syndrome when compared with controls.

The findings indicate that both glomerular and tubular function are altered in dogs with Cushing's syndrome. A longitudinal study to further investigate renal functional changes before and after treatment of hypercortisolism will be described in § 5.2.

## Introduction

Cushing's syndrome is one of the most common endocrine disorders, with an estimated incidence of 1 to 2 cases/1000 dogs/year.<sup>1</sup> In 80 to 85% of cases, chronic glucocorticoid excess is caused by increased adrenocorticotrophic hormone (ACTH) secretion by a pituitary tumor, while adrenal or ACTH-independent hypercortisolism accounts for about 15 to 20% of cases of spontaneous hypercortisolism in dogs.<sup>2</sup> Forty-four to 75% of dogs with untreated Cushing's syndrome have proteinuria and 59 to 86% have systemic hypertension.<sup>3-5</sup> Frequently, neither of these disease processes respond to treatment of hypercortisolism.<sup>3-4</sup> Proteinuria and hypertension are central to the development and progression of chronic kidney disease (CKD), and are associated with increased morbidity and mortality in dogs with naturally-occurring CKD.<sup>6-8</sup> Dogs with Cushing's syndrome may thus be at risk of developing renal complications. In human patients with Cushing's syndrome, decreased GFR and CKD, focal segmental glomerulosclerosis, nephrotic syndrome and an increased prevalence of nephrolithiasis have been observed.<sup>9-12</sup> Despite these associations, effects of spontaneous Cushing's syndrome on renal function remain poorly documented in humans as well as in dogs.<sup>13-14</sup>

Routine diagnostic markers such as creatinine and urea allow detection of kidney dysfunction only when at least 75% of renal functional mass is lost.<sup>15</sup> Measurement of GFR is considered a more sensitive index of renal function.<sup>16</sup> Additionally, new urinary markers, proposed for early and site-specific detection of renal dysfunction in human studies, are gaining interest in companion animal medicine.<sup>17-20</sup> They include high molecular weight (HMW) proteins such as immunoglobulin G (IgG), intermediate weight proteins such as albumin (ALB), low molecular weight proteins such as retinol-binding protein (RBP) and urinary enzymes such as N-acetyl- $\beta$ -D-glucosaminidase (NAG). Glomerular dysfunction causes increased filtration of IgG and ALB, whereas tubular dysfunction leads to excessive urinary excretion of RBP and NAG, due to disturbed reabsorption or increased protein trafficking and structural damage of tubule cells, respectively.<sup>21</sup> Several studies in dogs and cats demonstrated these markers' ability to detect subclinical renal dysfunction secondary to infectious or endocrine disorders, e.g. pyometra, leishmaniasis and hyperthyroidism.<sup>20,22-24</sup>

This study was established to assess renal function in dogs with Cushing's syndrome prior to treatment compared with healthy control dogs, using routine variables as well as GFR, glomerular and tubular urinary markers.

## Material and methods

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### *Animals*

This study was performed at the Faculty of Veterinary Medicine of Ghent University (Belgium) and the University Clinic for Companion Animals of Utrecht University (The Netherlands), after approval by the Local Ethical Committee of both institutions (approval numbers 2008/066 and 2008.III.06.052). All owners signed informed consent prior to inclusion.

Twenty-five client-owned dogs with Cushing's syndrome were included in the study (Cushing group). Cushing's syndrome was diagnosed based on history, clinical signs, physical examination, biochemical changes and consistent result on a combination of screening methodologies: low dose dexamethasone suppression test (LDDST), urinary corticoid-to-creatinine ratio (UCCR) in two consecutive morning urine samples, or ACTH-stimulation test. Pituitary-dependent hypercortisolism (PDH) was diagnosed when at least two of the following five test results were present: LDDST results indicative for PDH, UCCR suppression of 50% or more after oral administration of dexamethasone, increased plasma ACTH concentration, ultrasonographic evidence of two equal-sized adrenal glands or presence of a pituitary mass on computed tomography. Adrenal tumor hypercortisolism (ATH) was diagnosed when the LDDST result showed no suppression or when the UCCR was not suppressed after oral administration of dexamethasone, in combination with ultrasonographic evidence of an adrenal mass. A complete blood count, biochemistry profile and urinalysis including bacterial culture were performed in all dogs. Exclusion criteria were presence of concurrent systemic infectious, neoplastic, or endocrine diseases. Dogs treated with drugs affecting kidney function were also excluded.

Twelve privately-owned healthy dogs, aged seven years or older, were recruited as control subjects (control group). Dogs were judged healthy based on history, physical examination, complete blood count, biochemistry profile and urinalysis (negative bacterial culture). Recent administration of drugs possibly influencing renal function (e.g. non-steroidal anti-inflammatory drugs, glucocorticoids) was an exclusion criterion.

To avoid any potential confounding effect due to size-dependent variations in GFR, the number of healthy dogs was matched proportionally to the number of dogs with Cushing's syndrome in each of the following bodyweight classes: small (< 10 kg), medium (10-25 kg), large (> 25-45 kg) and giant (> 45 kg). Two Cushing dogs and one control dog weighed < 10 kg (small), 10 Cushing dogs and four controls weighed between 10 and 25 kg (medium), 11 Cushing dogs and six healthy dogs weighed more than 25 to 45 kg (large), and two Cushing dogs and one control dog weighed more than 45 kg (giant).



### ***Sampling procedures***

All dogs were fasted for at least 10 h prior to the test day and fed immediately after ending the sampling period. Water was provided *ad libitum*. Systolic blood pressure (SBP) was measured indirectly by Doppler method<sup>a</sup> after acclimatization to the environment, according to the American College of Veterinary Internal Medicine guidelines.<sup>25</sup> Five consistent consecutive measurements were averaged. Morning urine samples were taken by cystocentesis (10 mL, 22 G needle). Urinary dipstick analysis<sup>b</sup>, urine specific gravity (USG), urine protein-to-creatinine ratio (UPC), sediment analysis and bacterial culture were performed. After centrifugation, the urine supernatant was stored at  $-80^{\circ}\text{C}$  until analysis of urinary markers.

Glomerular filtration rate was measured by plasma clearance of exo- and endo-iohexol ( $\text{Cl}_{\text{exo}}$  and  $\text{Cl}_{\text{endo}}$ ), using a protocol adapted from van Hoek and coworkers.<sup>26</sup> Briefly, a 15 mL blood sample was collected from the jugular vein (22 G needle) for hematology and biochemistry, including creatinine and urea. A 22 G catheter was placed in the cephalic vein, 64.7 mg/kg (0.1 mL/kg) iohexol<sup>c</sup> was injected and a timer was started at the end of the injection. Afterwards, dead space in the catheter was rinsed with 3 mL of sodium chloride 0.9%. The catheter was removed and 2.5 mL EDTA blood samples were collected from the jugular vein at 5, 15, 60, 120, 240, 360 and 480 min after injection. Samples were centrifuged within 2 h and stored in aliquots of 300  $\mu\text{L}$  at  $-20^{\circ}\text{C}$  until assayed.

### ***Exo- and endo-iohexol assays***

Plasma concentrations of exo- and endo-iohexol were determined by high-performance liquid chromatography with ultraviolet detection, as previously validated and described.<sup>26</sup> Percentage of exo- and endo-iohexol measured in the Omnipaque<sup>®</sup> solution were 83.5% and 16.5%, respectively. This ratio was used to determine the dose of each isomer for clearance calculations. Plasma concentrations below the limit of quantification (LOQ) were not taken into account. The LOQ was 0.06 and 0.43  $\mu\text{g}/\text{ml}$  for endo- and exo-iohexol, respectively.

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### *Pharmacokinetic analysis*

Pharmacokinetic analyses were performed using WinNonlin<sup>d</sup>. For clearance calculation, individual plasma data were subjected to noncompartmental analysis, as described by Watson and coworkers.<sup>16</sup> The area under the plasma exo- and endo-iohexol concentration versus time curve (AUC) was calculated using the trapezoidal rule with extrapolation to infinity. Plasma  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$  were determined by dividing the actually administered dose of respectively exo- and endo-iohexol by the corresponding AUC, and indexed to bodyweight (mL/min/kg).

### *Urinary marker assays*

Urinary ALB and uIgG concentrations were determined with a commercial canine specific enzyme-linked immunosorbent assay (ELISA)<sup>e</sup>, uRBP with a human ELISA<sup>e</sup> and uNAG activity using a colorimetric assay<sup>f</sup>, as previously described and validated by our group.<sup>19,27</sup> Because spot urine samples were used, urinary concentration of each marker was indexed to urinary creatinine (c) and expressed as a ratio.<sup>28</sup> Urinary creatinine concentration was measured by a modified Jaffé reaction using picric acid.

### *Statistical analysis*

Analyses were performed with a commercial software program<sup>g</sup>. Results are expressed as median and range. Bodyweight, age, SBP, screat, urea, UPC, USG, uALB/c, uIgG/c, uRBP/c, uNAG/c,  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$  were compared between Cushing patients and healthy dogs using a Mann-Whitney *U*-test with a significance level of 5%. Results are expressed as median (range).

## Results

### *Dogs*

Most frequently represented breeds in the Cushing group were: Beagle (4/25), Boxer (3/25), Basset breeds (one Grand Basset Griffon Vendéen, one Basset Hound, one Basset Fauve de Bretagne) and mixed breed dogs (3/25). Typical clinical signs included polyuria and polydipsia (24/25), polyphagia (19/25), skin abnormalities (18/25), pot-belly (16/25), muscle weakness (10/25) and exercise intolerance (10/25). Pituitary-dependent hypercortisolism was diagnosed in 22/25 and ATH in 3/25 dogs. A Briard was diagnosed with combined ATH and CKD and one Beagle had concomitant PDH and CKD. One dog with PDH had a positive urine culture (*Escherichia coli*) and another dog with PDH had renal glucosuria. Age, body weight, and gender of the Cushing and control groups are presented in Table 1.

### *Renal variables*

Systolic blood pressure and renal variables in the Cushing and control group are presented in Table 1. When the Cushing group was compared to the control group, creat ( $p = 0.003$ ) and USG ( $p < 0.001$ ) were significantly decreased, and UPC ( $p < 0.001$ ) was significantly increased in the dogs with Cushing's syndrome. Urea ( $p = 0.85$ ) and SBP ( $p = 0.49$ ) were not different between groups. Urinary ALB/c, uIgG/c, uRBP/c and uNAG/c were significantly higher in the Cushing group than in healthy dogs ( $p < 0.001$ ).

Plasma exo- and endo-iohexol concentration-time curves in the Cushing and control groups are shown in Figure 1. Plasma exo-iohexol concentration (expressed as mean  $\pm$  SD) in each dog over the entire sampling period represents  $76.4 \pm 7.2$  % to  $87.9 \pm 2.2$  % of the overall concentration of iohexol, i.e. a ratio similar to that observed in the iohexol formulation. Plasma  $Cl_{\text{endo}}$  ( $p = 0.046$ ), but not  $Cl_{\text{exo}}$  ( $p = 0.416$ ), was significantly higher in the Cushing group (Table 1). The two dogs with concurrent CKD and PDH or ATH, had a  $Cl_{\text{exo}}$  of 1.1 and 0.5 ml/min/kg, and a  $Cl_{\text{endo}}$  of 0.9 and 0.4 ml/min/kg, respectively.

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**Table 1. Details of the descriptive statistics (median, range) for the Cushing and control group.**

\*  $P < 0.05$  between Cushing and control group.

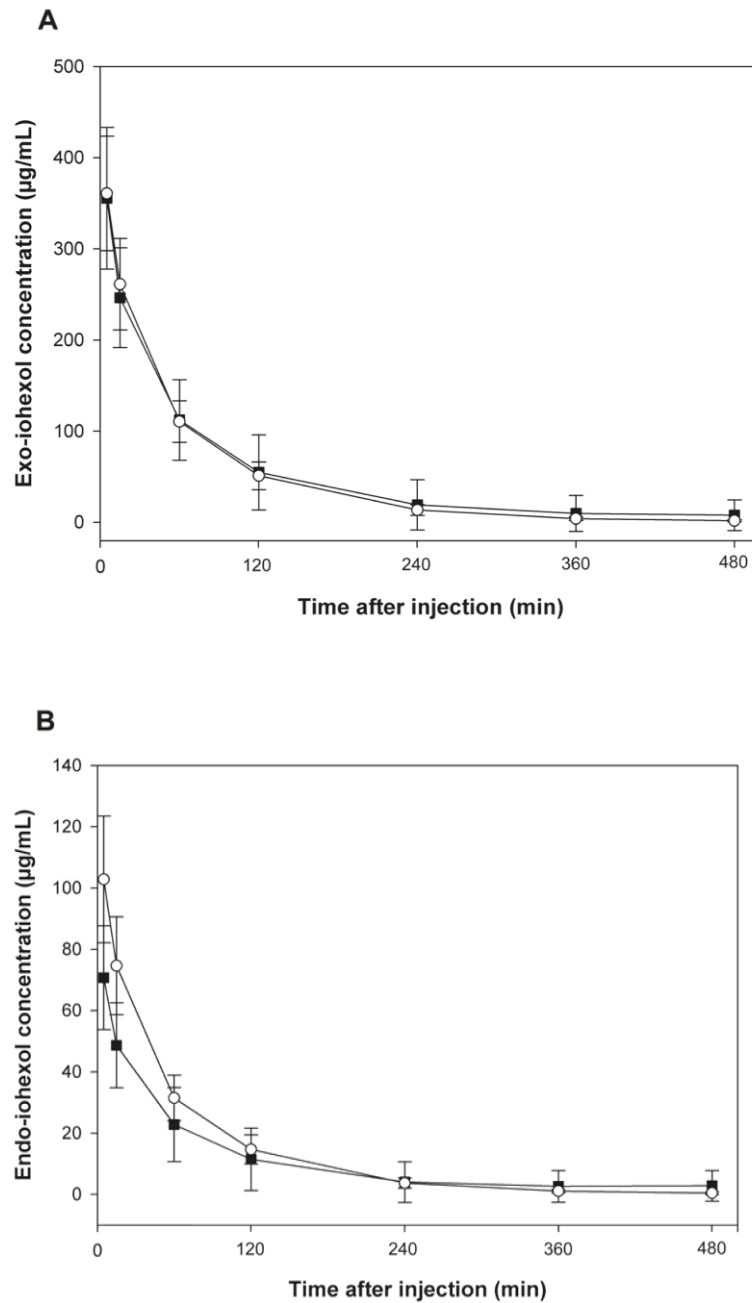
\*\*  $P < 0.001$  between Cushing and control group.

F, female (intact); FN, female (neutered); M, male (intact); MN, male (neutered). SBP, systolic blood pressure; screat, serum creatinine; USG, urine specific gravity; UPC, urine protein-to-creatinine ratio; uALB/c, urinary albumin-to-creatinine ratio; uIgG/c, urinary immunoglobulin G-to-creatinine ratio; uRBP/c, urinary retinol-binding protein-to-creatinine ratio; uNAG/c, urinary N-acetyl- $\beta$ -D-glucosaminidase-to-creatinine ratio;  $Cl_{exo}$ , plasma clearance of exo-iohexol;  $Cl_{endo}$ , plasma clearance of endo-iohexol

<b>Variable</b>	<b>Cushing group</b>	<b>Control group</b>
<b>Number of dogs</b>	25	12
<b>Age (years)</b>	10.6 (6.7-13.3)	8.5 (7-10.9)
<b>Bodyweight (kg)</b>	25.1 (4.1-56.5)	29.6 (9.5-51.2)
<b>Sex</b>	4 F, 9 FN, 10 M, 2 MN	2 F, 4 FN, 3 M, 3 MN
<b>SBP (mmHg)</b>	166 (103-230)	165 (102-200)
<b>screat (<math>\mu\text{mol/L}</math>)</b>	60 (35-182)*	84 (67-132)*
<b>urea (mmol/L)</b>	4.7 (2-19.5)	5.4 (3.2-7.3)
<b>USG</b>	1.008 (1.004-1.015)**	1.033 (1.016-1.050)**
<b>UPC</b>	3.4 (0.01-16.8)**	0.1 (0.06-0.19)**
<b>uALB/c (mg/g)</b>	2593 (45.4-15717.3)**	9.6 (2.3-152.6)**
<b>uIgG/c (mg/g)</b>	521.6 (4.2-3468.2)**	2.7 (0.8-17.7)**
<b>uRBP/c</b>	2.6 (0.2-485.6)**	0.06 (0-0.89)**
<b>uNAG/c (U/g)</b>	8.6 (1.8-31.8)**	2.0 (1.2-5.2)**
<b><math>Cl_{exo}</math> (mL/min/kg)</b>	2.3 (0.5-3.7)	2.2 (1.7-3.6)
<b><math>Cl_{endo}</math> (mL/min/kg)</b>	2.7 (0.4-5.1)*	1.9 (1.3-2.8)*

**Figure 1. Plasma concentration-time curves of exo-iohexol (A) and endo-iohexol (B) in dogs with Cushing's syndrome (black squares) and in control dogs (white circles).**

Plasma concentrations are expressed as mean  $\pm$  SD.



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### Discussion

This is the first study to determine GFR and urinary marker concentrations in dogs with Cushing's syndrome. Results indicate altered glomerular and tubular function in dogs with Cushing's syndrome, as evidenced by increased UPC, uALB/c, uIgG/c, uRBP/c and uNAG/c compared to healthy dogs. Plasma  $Cl_{\text{endo}}$ , but not  $Cl_{\text{exo}}$  was higher in the Cushing group than in the control dogs.

Regarding routine renal variables, results were similar to previous studies. Serum creatinine was decreased by 29% in the Cushing group, as previously reported.<sup>29</sup> Not only renal function affects sreat concentration, but also production of creatinine from creatine in skeletal muscle, as shown in dogs with CKD<sup>16</sup> and with experimental hypothyroidism.<sup>30-31</sup> In Cushing patients, both decreased muscle mass and increased GFR might contribute to a lower sreat. Urea is usually within or in the lower part of the reference range in dogs with Cushing's syndrome.<sup>32</sup> Urinary specific gravity was also significantly lower in the Cushing patients, as a result of polyuria and polydipsia. Excess cortisol indeed disturbs osmoregulation of vasopressin release and causes resistance to its renal action, leading to a decreased urinary concentrating ability.<sup>33-34</sup>

In the Cushing group, UPC was markedly increased compared to controls. This result is comparable to previously reported mean UPCs of 1.47 in dogs with untreated PDH, 2.7 in dogs with untreated ATH and an overall mean of 1.9 in dogs with either PDH or ATH.<sup>5,35</sup> In our study, the highest UPC was 16.8 in a dog with concomitant PDH and CKD, although one dog with only ATH and two dogs with only PDH also had surprisingly high UPC's (10.16, 10.05 and 16.32, respectively), with a normal urine sediment. It is difficult, however, to elucidate whether this proteinuria is related solely to the Cushing's syndrome or to underlying glomerular disease.

Renal proteinuria can be caused by increased glomerular permeability, impaired tubular reabsorption of filtered protein, or both. Marked increases in UPC, uALB/c and uIgG/c in the diseased group indicates proteinuria of glomerular origin which may result from glomerular hypertension or altered glomerular permselectivity. High uIgG/c suggests the latter, since the glomerular barrier is normally impermeable to this HMW protein.<sup>36</sup> Although the SBP was not increased in the Cushing compared to the control group, this does not preclude the possibility that glomerular hypertension contributed to the proteinuria.<sup>3,5</sup> Despite

the measures taken to reduce stress-induced hypertension, we cannot completely exclude the influence of handling stress in dogs of both groups.

Furthermore, increased uRBP/c and uNAG/c in the Cushing group indicate proteinuria of tubular origin. Glomerular-filtered RBP is reabsorbed through megalin-mediated endocytosis by proximal tubular cells, causing urine of healthy dogs to contain very little RBP.<sup>37-38</sup> Increased urinary excretion of RBP, associated with tubular dysfunction, has been described in humans and dogs with CKD, in dogs with pyometra and in hyperthyroid cats.<sup>19-20,24,38-39</sup> This may result from impairment of megalin-mediated endocytosis due to tubulo-interstitial damage.<sup>38</sup>

Urinary NAG/c may be increased because of tubular damage and leakage from tubular cell lysosomes or higher lysosomal activity due to increased protein processing, as was demonstrated in rats.<sup>40</sup> In humans with glomerulonephritis, in geriatric cats with azotemia and in dogs with CKD, it has been demonstrated that uNAG/c is correlated with UPC and/or albuminuria, but not with sreat concentration in dogs and cats, or with GFR in humans.<sup>19,41-42</sup> Therefore increased uNAG/c may reflect alteration of tubular function rather than tubular damage. In people, the isoenzyme NAG A is physiologically excreted in urine by exocytosis, whereas NAG B is part of the lysosomal membrane and released when there is actual damage to the tubular cell.<sup>43</sup> Identification of similar NAG isoenzymes in the dog could contribute to differentiation between upregulated NAG activity and tubular cell damage.

Plasma  $Cl_{\text{exo}}$  was not significantly different between the two groups of dogs, whereas  $Cl_{\text{endo}}$  was higher in the Cushing group. Differences in plasma clearance of the two stereoisomers have previously been described and indicate that dispositions of exo- and endo-iohexol may vary in different clinical settings for reasons that currently remain unclear.<sup>44</sup> The dose calculation of both isomers is unlikely to be responsible for the observed differences, since the exo/endo-iohexol ratio in plasma was similar to that observed in the iohexol formulation. The only way to identify the underlying mechanisms would be to perform specific pharmacokinetic studies by administering each stereoisomer separately. A direct consequence is that measurement of total iohexol is misleading for GFR assessment, since it is the sum of two stereo-isomers with different kinetics.

Exo-iohexol is the major stereo-isomer and is typically considered a marker of GFR in dogs.<sup>45</sup> No statistically significant difference in plasma  $Cl_{\text{exo}}$  was observed between the Cushing and control group. A previous pilot study in a smaller group of dogs with Cushing's

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syndrome showed a higher plasma  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$  compared to control dogs.<sup>46</sup> However, bodyweight may have been a confounding factor in the latter study, because dogs of both groups were not bodyweight matched. Plasma clearance of iohexol can indeed be higher in small dogs compared to large dogs.<sup>47</sup> Another explanation could be the duration of exposure to high plasma cortisol concentrations. Short term administration of ACTH or glucocorticoids increases GFR in rats, dogs and humans.<sup>48-50</sup> On the contrary, long-term effects of Cushing's syndrome in human patients may decrease GFR, as shown in a study by Haentjens and coworkers<sup>12</sup>, in which four out of 18 patients were identified with CKD.

Urinary tract infection (UTI) was an exclusion criterion for the healthy dogs, but one dog with Cushing's syndrome had a positive urine culture (*E. coli*). Information about effects of UTI on urinary markers in dogs is scarce. Experimentally induced cystitis increased UPC in one study, and another study demonstrated that not pyuria alone, but the combination of pyuria, hematuria and bacteriuria leads to higher urinary albumin concentrations.<sup>51-52</sup> The effect of lower UTI on uNAG/c seems to be minimal in dogs, but an increased uNAG/c ratio is reported in the presence of pyelonephritis.<sup>53</sup> To the authors' knowledge, the effect of UTI on uRBP/c and uIgG/c is largely unknown in dogs. In one pilot experiment, addition of white blood cells, red blood cells and bacteria to urine samples did not affect uALB/c, uRBP/c and uNAG/c, but this does not fully mimic an *in vivo* inflammatory response.<sup>54</sup>

### Conclusion

This study found evidence of both renal glomerular and tubular dysfunction in dogs with untreated Cushing's syndrome. A longitudinal study to further investigate renal functional changes before and after treatment of hypercortisolism will be described in § 5.2.

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**Sources and manufacturers**

<sup>a</sup>Parks Medical Electronics Inc., Aloha, OR, USA

<sup>b</sup>Combur stick, Roche Diagnostics, Burgess Hill, UK

<sup>c</sup>Omnipaque 300, GE Healthcare, Amersham Health, Wommel, Belgium

<sup>d</sup>WinNonlin version 4.0.1, Pharsight

<sup>e</sup>Dog albumin, Dog IgG and Human RBP ELISA kit, Immunology Consultants Laboratory,  
Newberg, OR, USA

<sup>f</sup> $\beta$ -N-Acetylglucosaminidase Assay kit, Sigma-Aldrich, St.-Louis, MO, USA

<sup>g</sup>Systat, version 12.00.08

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**§ 5.2 LONG-TERM FOLLOW-UP OF RENAL FUNCTION IN DOGS WITH  
CUSHING'S DISEASE BEFORE AND AFTER TREATMENT**

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### Summary

Systemic arterial hypertension and proteinuria are frequent complications in dogs with Cushing's syndrome and do not always resolve after treatment of hypercortisolism. Therefore, dogs with Cushing's syndrome may be at risk for renal dysfunction before and after treatment. Objectives were to assess renal function in dogs with pituitary-dependent hypercortisolism (PDH) before and after treatment.

Nineteen dogs with PDH and 12 control dogs were included in the study. Renal function was assessed before, and at 1, 3, 6 and 12 months after treatment. Twelve dogs were treated with trilostane and 7 dogs by transsphenoidal hypophysectomy. Routine renal markers were measured and urinary albumin (uALB), immunoglobulin G (uIgG), and retinol-binding protein (uRBP) were assessed by ELISA. Urinary N-acetyl- $\beta$ -D-glucosaminidase (uNAG) was determined colorimetrically. All urinary markers were indexed to urinary creatinine concentration (c). Plasma clearance of creatinine ( $Cl_{\text{creat}}$ ), exo-iohexol ( $Cl_{\text{exo}}$ ), and endo-iohexol ( $Cl_{\text{endo}}$ ) were used to measure GFR. Data were analyzed using a general linear model.

Serum creatinine and urea increased post-treatment, but remained within reference ranges. Plasma  $Cl_{\text{creat}}$  and  $Cl_{\text{endo}}$  were significantly lower post-treatment, whereas  $Cl_{\text{exo}}$  was not different. Urinary protein-to-creatinine ratio (UPC), uALB/c, uIgG/c and uRBP/c were decreased post-treatment, but at 12 months 5/13 dogs remained proteinuric. Urinary NAG/c did not change significantly.

Post-treatment GFR decline and persistent proteinuria may warrant the clinician's attention. Future research including kidney histopathology of patients with persistent proteinuria or a low GFR is needed to further assess renal outcome.



## Introduction

Cushing's syndrome is a frequent endocrine disorder in middle-aged and older dogs,<sup>1</sup> and is treated with trilostane, a reversible inhibitor of cortisol synthesis, or by transsphenoidal hypophysectomy.<sup>2-3</sup> Up to 86% of dogs with untreated Cushing's syndrome have systemic arterial hypertension and 44-75% have proteinuria, which may persist despite successful treatment of hypercortisolism.<sup>4-6</sup> Proteinuria and systemic arterial hypertension are major factors in development and progression of chronic kidney disease (CKD) and associated with increased morbidity and mortality in dogs with CKD.<sup>7-9</sup> Therefore, dogs with Cushing's syndrome could be considered at risk for renal complications. In human patients with Cushing's syndrome, decreased glomerular filtration rate (GFR), focal segmental glomerulosclerosis, nephrotic syndrome and an increased prevalence of nephrolithiasis have been reported.<sup>10-13</sup> However, effects of spontaneous hypercortisolism on renal function is poorly documented in humans and dogs.<sup>14-15</sup>

Use of sensitive and direct methods is needed to detect subtle changes in renal function. Measurement of GFR is considered the best overall indicator of renal function.<sup>16</sup> In addition, urinary markers may allow early and site-specific detection of renal dysfunction.<sup>17-20</sup> These include high molecular weight (HMW) proteins, e.g. immunoglobulin G (IgG), intermediate molecular weight proteins (IMW), e.g. albumin (ALB), low molecular weight (LMW) proteins, e.g. retinol-binding protein (RBP) and urinary enzymes, e.g. N-acetyl- $\beta$ -D-glucosaminidase (NAG). Glomerular dysfunction causes increased filtration of IgG and ALB, whereas tubular dysfunction leads to increased urinary excretion of RBP and NAG.<sup>21</sup> These markers allow detection of subclinical renal dysfunction secondary to infectious or endocrine disorders in dogs and cats, e.g. pyometra<sup>22-23</sup>, leishmaniasis<sup>24</sup> and hyperthyroidism.<sup>25</sup> Urine (u) ALB-to-creatinine (c) ratio (uALB/c), uIgG/c, uRBP/c and uNAG/c are increased in untreated dogs with Cushing's syndrome compared to 12 healthy controls.<sup>26</sup>

This prospective longitudinal study aimed to assess renal function in dogs with pituitary-dependent hypercortisolism (PDH) before and after medical or surgical treatment of hypercortisolism, using routine renal variables as well as GFR, glomerular and tubular urinary markers.

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### Materials and methods

#### *Animals and study design*

This study was performed at the Faculty of Veterinary Medicine of Ghent University (Belgium) and the University Clinic for Companion Animals of Utrecht University (The Netherlands). Following approval by the Ethical Committee of both institutions (EC2008/066 and 2008.III.06.052) and informed owner consent, 19 client-owned dogs with pituitary-dependent hypercortisolism (PDH) were included in the study. Cushing's disease was suspected based on history, clinical signs, physical examination, biochemical changes and a result consistent with hypercortisolism on at least 1 of these screening tests: low dose dexamethasone suppression test (LDDST), urinary cortisol-to-creatinine ratio (UCCR) in 2 consecutive morning urine samples or ACTH-stimulation test. Diagnosis of PDH was confirmed when at least 2 of the following 5 test results were present: LDDST results indicative for PDH, UCCR suppression for 50% or more after oral administration of dexamethasone, increased plasma ACTH concentration, ultrasonographic evidence of 2 equal-sized adrenal glands or presence of a pituitary mass on computed tomography. Exclusion criteria were presence of concurrent systemic infectious, neoplastic, or endocrine diseases, and treatment with drugs potentially affecting kidney function. Cardiac disease was an exclusion criterion except for dogs with subclinical disease (International Small Animal Cardiac Health Council class Ia and Ib).

Twelve dogs (Mx group) were treated medically with trilostane<sup>a</sup> at a starting dose of 2 mg/kg every 24h (sid) or 1 mg/kg every 12h (bid). Response to treatment was based on control of clinical signs and an ACTH<sup>b</sup>-stimulation test. Dogs were considered to be adequately controlled when clinical signs were resolved and post-ACTH cortisol was between 40 and 150 nmol/L.

Seven dogs (Hx group) underwent a transsphenoidal hypophysectomy and received replacement therapy with cortisone acetate (1 mg/kg bid starting dose tapered over 4 weeks to 0.25 mg/kg bid), levothyroxine (15 µg/kg) and desmopressin (0.01% 1 drop into the conjunctival sac every 8h, tapered tailored to individual patient) (Hx group).<sup>27</sup> Response to treatment was assessed based on clinical signs and UCCRs with additional evaluation of serum total thyroxine concentration. Hypercortisolism was considered to be in remission when clinical signs were resolved and UCCR's were below  $8.3 \times 10^{-6}$ .<sup>28</sup> Choice of therapy relied on the owners' decision and randomization of the 2 treatment groups was not performed.

All dogs were evaluated 1 day before treatment (T0) and at 1 (T1), 3 (T3), 6 (T6) and 12 (T12) months after treatment. Tested variables at all time points were body weight (BW), systolic blood pressure (SBP), serum creatinine (screat), serum urea, urine specific gravity (USG), urine protein-to-creatinine ratio (UPC), uALB/c, uIgG/c, uRBP/c and uNAG/c. Complete blood count (CBC), biochemistry profile and urine cultures were performed at T0, T6 and T12, or for urine culture more often when indicated based on sediment analysis. At the end of the study, owners were interviewed by telephone to document clinical outcome of the patients.

Because control values for plasma clearance of creatinine ( $Cl_{\text{creat}}$ ), exo- and endo-iohexol ( $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$ ), are currently not well established and often different analytical methods are used, 12 healthy BW matched control dogs  $\geq 7$  years old were included for measurement of GFR. For  $Cl_{\text{creat}}$  a lower limit of 2 mL/min/kg has been previously suggested.<sup>29-31</sup> Dogs were judged healthy based on history, physical examination, CBC, biochemistry profile and urinalysis (negative bacterial culture). Recent administration of drugs possibly affecting renal function was an exclusion criterion.

### *Sampling procedures*

SBP was measured indirectly by Doppler method<sup>c</sup>, according to the American College of Veterinary Internal Medicine guidelines.<sup>32</sup> Morning urine samples were taken by cystocentesis. Urinary dipstick analysis<sup>d</sup>, USG, UPC, sediment analysis and bacterial culture were performed. After centrifugation, the urine supernatant was stored at  $-80^{\circ}\text{C}$  until assayed.

Glomerular filtration rate was simultaneously measured by  $Cl_{\text{creat}}$ ,  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$ , as described in a previous study and in paragraph 5.1.<sup>33</sup>

### *Assays and pharmacokinetic analysis*

Serum urea (reference interval 1.16-8.49 mmol/L) was measured using an enzymatic method<sup>f</sup>. Urinary protein was determined with a turbidimetric method using benzethonium chloride and urinary creatinine with a modified Jaffé reaction. Urinary ALB, IgG and RBP were determined with an ELISA<sup>g</sup> and uNAG with a colorimetric assay<sup>h</sup>, as previously validated, and indexed to urinary creatinine (c).<sup>17,34</sup>

Enzymatic analysis of plasma creatinine, and measurement of iohexol stereo-isomers (exo- and endo-iohexol) with high performance liquid chromatography were performed as previously described.<sup>29,33</sup> A noncompartmental analysis was performed using software<sup>i</sup> as

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previously described, with calculation of the area under the plasma creatinine, exo- and endo-iohexol concentration versus time curve (AUC).<sup>16</sup> Plasma  $Cl_{\text{creat}}$ ,  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$  were determined by dividing the administered dose by the corresponding AUC, and indexing to BW (mL/min/kg).

### *Statistical analysis*

Analyses were performed with commercial software<sup>1</sup>. Level of significance was set at 5%. A general linear model was used to test the effect of time on the selected variables. When a significant effect of time was detected, a Tukey post hoc hypothesis test was performed to analyze which time points differed significantly.

## Results

### *Dogs*

All 19 dogs were followed-up for at least 6 months. The baseline examination of most of these dogs was also used in the study in § 5.1. Thirteen of 19 dogs underwent the final check-up at T12 (7 dogs in the Mx group and 6 dogs in the Hx group). Between T6 and T12, 3 dogs died or were euthanized due to cardiac disease (n=1), central nervous signs (n=1) or severe respiratory disease (n=1) and 3 dogs were lost to follow-up because of owners withdrawing them from the study (n=3). At the time of submission 10/19 dogs had died or were euthanized because of non-renal related causes, with a mean survival time of 523 days.

At T0, mean  $\pm$  standard deviation (SD) age and BW were  $10.1 \pm 1.6$  years and  $27.2 \pm 11.4$  kg in the complete group,  $10.5 \pm 1.9$  years and  $24.8 \pm 12.1$  kg in the Mx group, and  $9.4 \pm 0.6$  years and  $31.3 \pm 9.8$  kg in the Hx group, respectively. Age and BW in the control group were  $8.7 \pm 1.6$  years and  $27.9 \pm 14.2$  kg. Bodyweight and SBP did not change significantly post-treatment (Table 1). Systolic BP was  $\geq 160$  mmHg in 7/19 dogs at T0, in 6/19 at T6 and in 5/13 dogs at T12.

In the Mx group, adequate control of PDH was achieved by T1. At T3, 1/12 dogs was poorly controlled and at T12 1/7 dogs had clinical signs caused by concurrent diabetes mellitus. Total starting dose of trilostane was  $1.8 \pm 0.5$  mg/kg bid in 9/12 dogs and  $2.4 \pm 0.2$  mg/kg sid in 3/12 dogs. At T12, trilostane dose was  $1.6 \pm 0.7$  mg/kg bid in 6/7 dogs and 3 mg/kg sid in 1 dog.

In the Hx group, control of PDH in all dogs was achieved by T3. At T6 2/7 dogs had recurrence of PDH with increased UCCRs ( $45.0$  and  $44.8 \cdot 10^{-6}$ ;  $5.0$  and  $7.4 \cdot 10^{-6}$ ). The latter two dogs were subsequently treated with trilostane with good clinical control at T12.

### *Post-treatment course of renal variables*

Descriptive statistics for the complete group are presented in Table 1. Screat was increased post-treatment in the complete group at T6 ( $p = 0.011$ ) and in the Hx group at T3 ( $p = 0.037$ ), but not in the Mx group. Two dogs had a mildly elevated screat ( $> 125$   $\mu\text{mol/L}$ ) at T0 and T6 ( $126$  and  $132$   $\mu\text{mol/L}$ , respectively). Urea was higher in the complete group and the Mx group at T1 ( $p = 0.020$  and  $p = 0.041$ , respectively) and T6 ( $p = 0.019$  and  $p = 0.007$ ), but did not differ post-treatment in the Hx group.

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Plasma  $Cl_{\text{creat}}$ ,  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$  in the control dogs were  $2.6 \pm 0.5$ ,  $2.4 \pm 0.6$ ,  $2.6 \pm 0.9$  mL/min/kg, respectively. Plasma  $Cl_{\text{creat}}$  was unavailable at baseline in 3/19 PDH patients. Pretreatment  $Cl_{\text{creat}}$ ,  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$  are presented in Table 1 and were higher than plasma clearance values of the controls in 10/16, 11/19 and 14/19 PDH patients, respectively. Post-treatment  $Cl_{\text{creat}}$  was significantly lower than pretreatment values at T6 and T12 in the complete group ( $p < 0.001$ ) and the Hx group ( $p = 0.001$  and  $p = 0.002$ , respectively) (Figure 1). In the Mx group,  $Cl_{\text{creat}}$  was decreased at T6 ( $p = 0.014$ ), but not at T12 (Figure 1). At T0 and T12, respectively, 1/16 and 4/13 dogs had a  $Cl_{\text{creat}}$  below the previously suggested lower limit of 2.0 mL/min/kg. There was a mild, non-significant post-treatment decline in  $Cl_{\text{exo}}$ , whereas  $Cl_{\text{endo}}$  was lower in the complete group at T12 ( $p = 0.024$ ) (Figure 1).

Post-treatment USG increased in the complete group and the Mx group at T3 ( $p = 0.005$  and  $p = 0.004$ ), T6 ( $p < 0.001$ ) and T12 ( $p < 0.001$ ), but there was no significant change in the Hx group. In all groups, UPC (Figure 1) decreased significantly after treatment ( $p < 0.001$ ) at all timepoints except for T1 and T12 in the Mx group. At T0, T6 and T12, UPC was above the cut-off ( $> 0.5$ ) in 13/19 (9/12 Mx, 4/7 Hx), 7/19 (5/12 Mx, 2/7 Hx) and 5/13 (3/7 Mx, 2/6 Hx) dogs, respectively. At T12, the 3 proteinuric dogs in the Mx group had UPCs of 0.78, 1.72 and 7.74. The latter dog was diagnosed with concurrent diabetes mellitus at T12. Previous blood and urinary examinations did not show hyperglycemia and glucosuria. The 2 dogs in the Hx group with mild proteinuria at T6 (0.62 and 0.72) and T12 (0.64 and 0.52) were those with recurrence of PDH at T6. At T12 they were treated with trilostane with good clinical control and optimal dosage as assessed by an ACTH-stimulation test.

A decrease in uALB/c at T3 ( $p = 0.015$ ) and T6 ( $p = 0.013$ ), in uIgG/c at T3 (0.0049) and in uRBP/c ( $p = 0.033$ ) at all time points in the complete group was observed (Figure 2), but inversely urine NAG/c did not change significantly. While uALB/c ( $p = 0.004$ ) and uIgG/c (0.002) were decreased in the Hx group at all time points and uRBP/c ( $p = 0.021$ ) at T1, T3 and T6, there was no statistically significant difference in these markers post-treatment in the Mx group.

**Table 1. Median (range) of tested variables pre- and post-treatment in 19 dogs with PDH.**

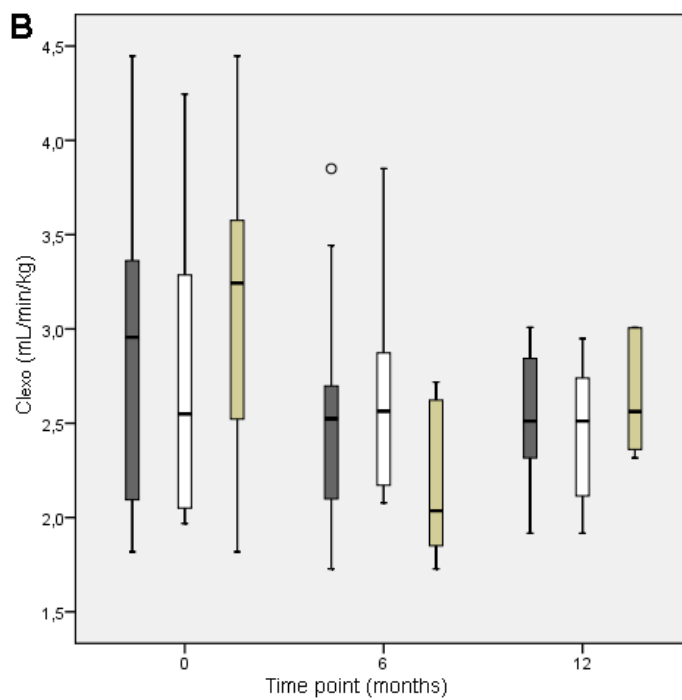
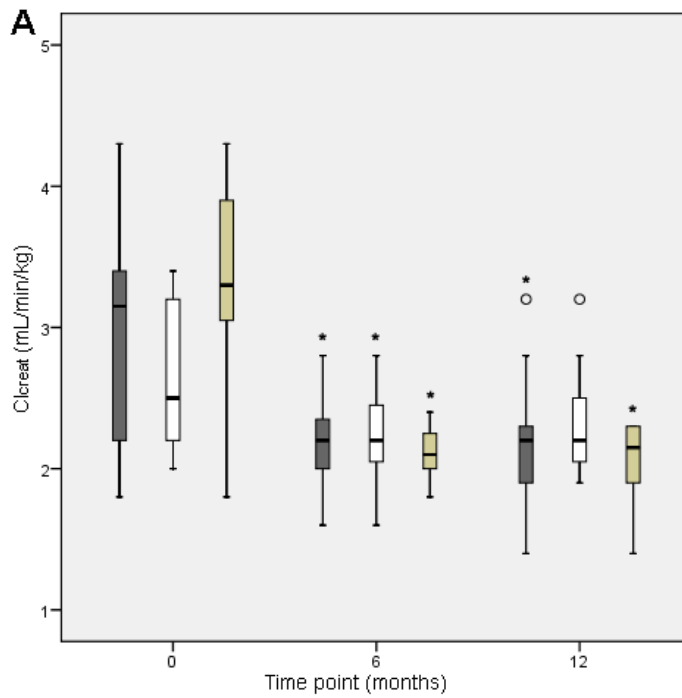
T0, pretreatment; T1, T3, T6, T12: 1, 3, 6 and 12 months post-treatment; SBP, systolic blood pressure; screat, serum creatinine; USG, urine specific gravity; UPC, urine protein-to-creatinine ratio; uALB/c, urinary albumin-to-creatinine ratio; uIgG/c urinary albumin-to-creatinine ratio uRBP/c, urinary retinol-binding protein-to-creatinine ratio; uNAG/c, urinary N-acetyl- $\beta$ -D-glucosaminidase-to-creatinine ratio;  $Cl_{creat}$ , plasma clearance of creatinine;  $Cl_{exo}$  plasma clearance of exo-iohexol;  $Cl_{endo}$ , plasma clearance of endo-iohexol; NA, not assessed; \* indicates a significant difference versus T0 ( $p < 0.05$ ).

Tested variable	Time point				
	T0	T1	T3	T6	T12
BW (kg)	28.2 (9.5-47.0)	28.0 (9.6-47.7)	28.0 (9.6-48.6)	29.3 (9.2-48.7)	30.2 (13.9-45.2)
SBP (mmHg)	155 (103-230)	138 (122-180)	155 (124-207)	140 (110-220)	155 (120-195)
screat ( $\mu$ mol/L)	53 (37-126)	62 (46-117)	63 (44-119)	65* (48-132)	67 (56-100)
urea (mmol/L)	3.8 (2.0-8.3)	4.3* (3.2-8.0)	4.0 (2.3-7.3)	4.7* (2.7-6.7)	4.5 (1.3-6.2)
USG	1.009 (1.003-1.022)	1.011 (1.003-1.025)	1.013* (1.004-1.039)	1.023* (1.004-1.039)	1.020* (1.002-1.045)
UPC	1.66 (0.01-16.32)	0.41* (0.06-4.96)	0.27* (0.06-4.01)	0.25* (0.09-4.7)	0.28* (0.08-7.47)
uALB/c (mg/g)	951.44 (31.19-13713)	263.39 (2.03-6659.04)	131.65* (1.45-4483.08)	129.60* (3.47-5236.51)	159.54 (4.73-12903.48)
uIgG/c (mg/g)	189.35 (0.71-6186.11)	17.59 (0-1410.03)	12.1* (0.75-700.33)	24.74 (1.18-712.93)	27.03 (1.29-1365.10)
uRBP/c (mg/g)	0.63 (0.21-37.87)	0.10* (0-1.59)	0.12* (0-1.35)	0.11* (0-0.82)	0.10* (0-4.77)
uNAG/c (mg/g)	4.03 (0-58.30)	2.27 (0-58.45)	2.39 (0-22.57)	3.19 (0-13.56)	3.21 (0-11.78)
$Cl_{creat}$ (mL/min/kg)	3.2 (1.8-4.3)	NA	NA	2.2* (1.6-2.8)	2.2* (1.4-3.2)
$Cl_{exo}$ (mL/min/kg)	3.0 (1.8-4.5)	NA	NA	2.5 (1.7-3.9)	2.5 (1.9-3.0)
$Cl_{endo}$ (mL/min/kg)	2.8 (1.9-4.9)	NA	NA	2.6 (1.6-4.3)	2.3* (1.8-3.2)

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**Figure 1. Post-treatment course of  $Cl_{creat}$  (A),  $Cl_{exo}$  (B),  $Cl_{endo}$  (C) and UPC (D) in the complete group (n=19), and subgroups of dogs that were treated with trilostane (n=12) or hypophysectomy (n=7).**

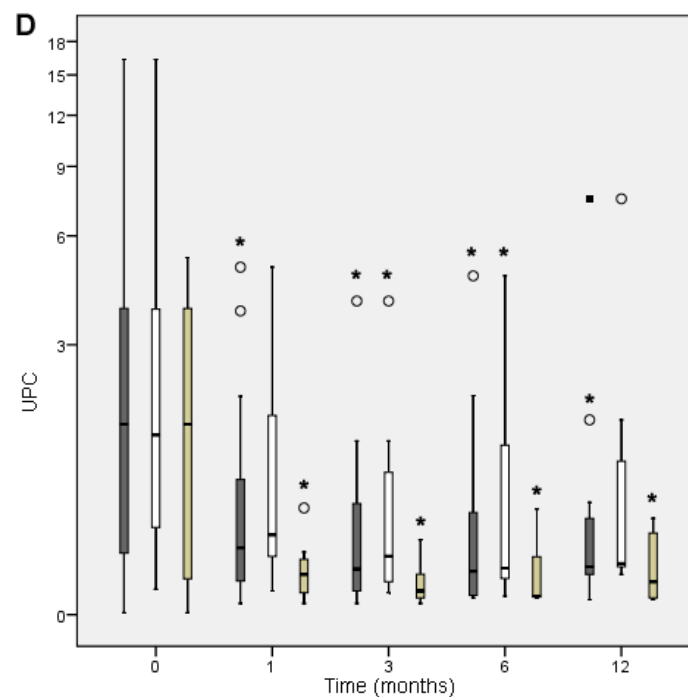
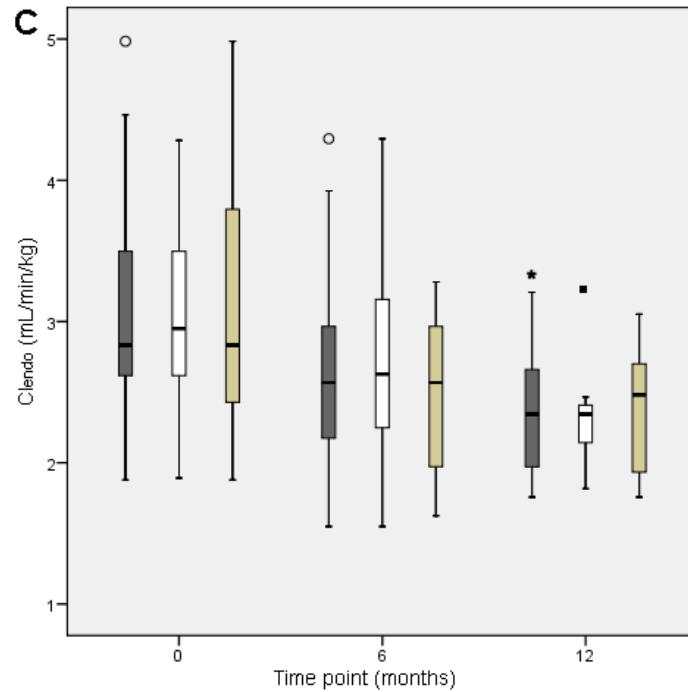
Dark grey box: complete group, white box: trilostane group, yellow box, hypophysectomy group. Box: interquartile range, o: outlier, \*: significant difference ( $p < 0.05$ ) compared to time point 0.





**Figure 1 (continued).** Post-treatment course of  $Cl_{creat}$  (A),  $Cl_{exo}$  (B),  $Cl_{endo}$  (C) and UPC (D) in the complete group (n=19), and subgroups of dogs that were treated with trilostane (n=12) or hypophysectomy (n=7).

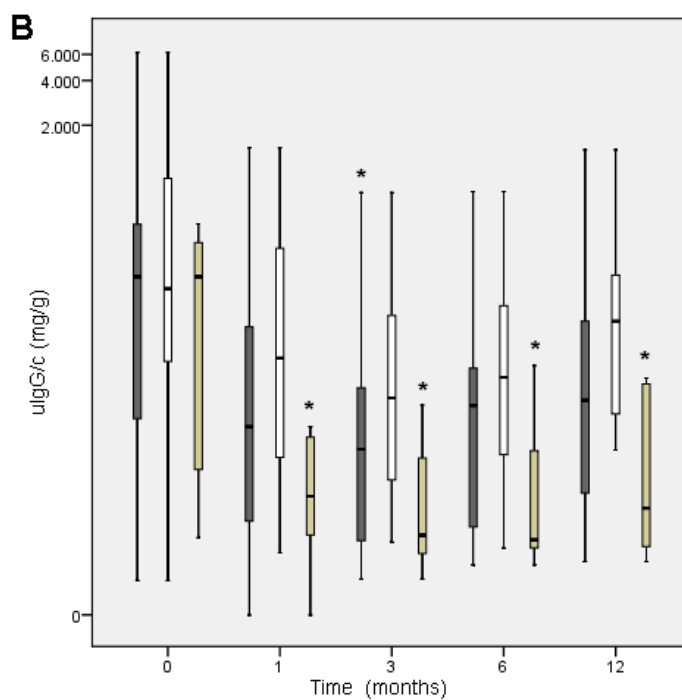
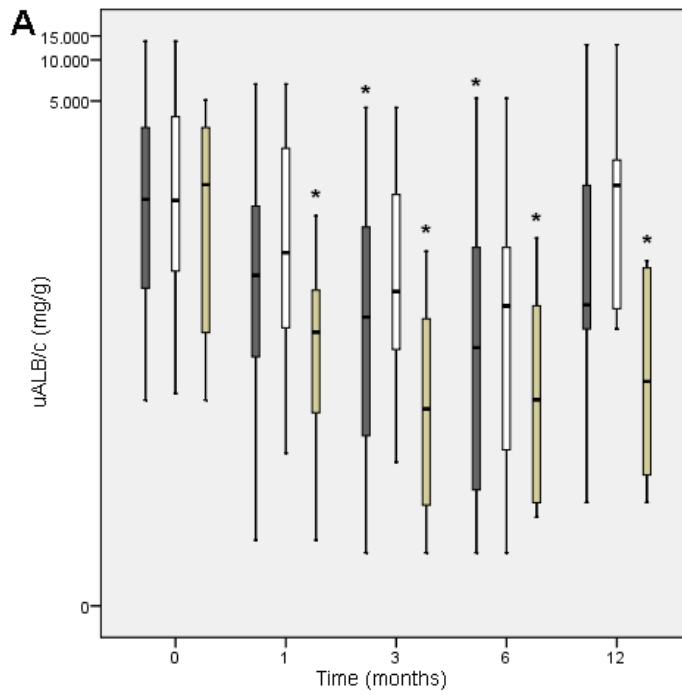
Dark grey box: complete group, white box: trilostane group, yellow box, hypophysectomy group. Box: interquartile range, o: outlier, black square: extreme value, \*: significant difference ( $p < 0.05$ ) compared to time point 0.



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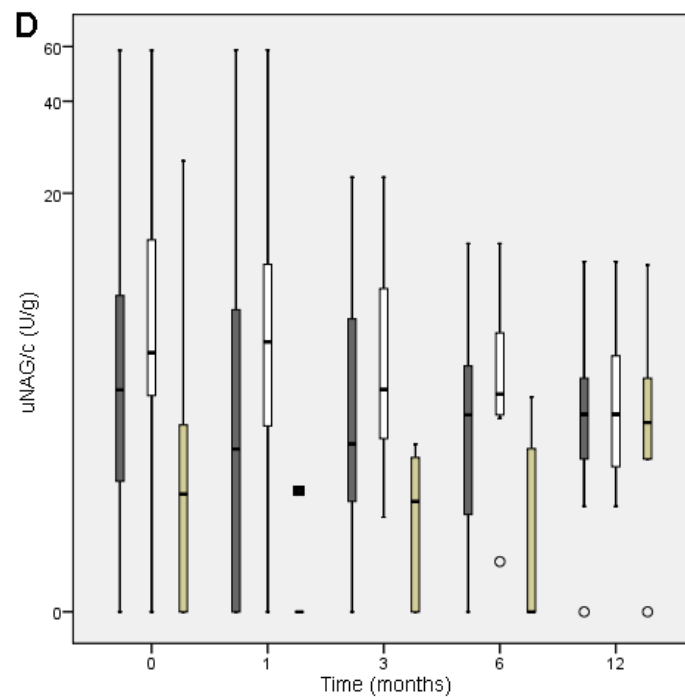
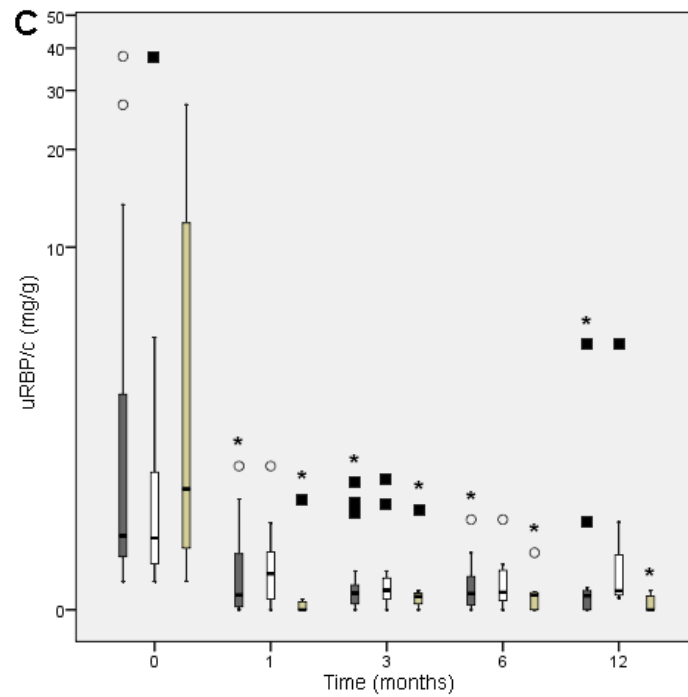
**Figure 2. Post-treatment course of uALB/c (A), uIgG/c (B), uRBP/c (C) and uNAG/c (D) in the complete group (n=19), and subgroups of dogs that were treated with trilostane (n=12) or hypophysectomy (n=7).**

Dark grey box: complete group, white box: trilostane group, yellow box, hypophysectomy group. Box: interquartile range, o: outlier, \*: significant difference ( $p < 0.05$ ) compared to time point 0.



**Figure 2 (continued).** Post-treatment course of uALB/c (A), uIgG/c (B), uRBP/c (C) and uNAG/c (D) in the complete group (n=19), and subgroups of dogs that were treated with trilostane (n=12) or hypophysectomy (n=7).

Dark grey box: complete group, white box: trilostane group, yellow box, hypophysectomy group. Box: interquartile range, o: outlier, black square: extreme value, \*: significant difference ( $p < 0.05$ ) compared to time point 0.



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Post-mortem kidney histopathological examination was available for two dogs that had died between T6 and T12. The first dog was an 11 year old Boxer that died of a ventricular tachycardia. Light microscopic evaluation revealed mild thickening of Bowman's capsule, tubular deposits (presumably protein) and focal accumulation of lymphocytes, plasma cells and macrophages. The second dog was a 13 year old Jack Russell Terrier euthanized because of severe respiratory distress (diffuse interstitial pneumonia). Kidney histopathology showed diffuse glomerular lesions including mild hypercellularity, thickening of the glomerular basement membrane and granular deposits in the glomerular tuft. Multifocal cystic glomerular atrophy and glomerulosclerosis were also found. Tubulo-interstitial lesions included tubular atrophy, thickening of the tubular basement membrane, mild interstitial fibrosis and few mononuclear cell infiltrates.

## Discussion

This study is the first to prospectively assess long-term effects of canine PDH on renal function, using both routine renal markers and more sensitive tests such as GFR and urine markers. Main findings are that GFR is increased pretreatment and decreases post-treatment. Furthermore, UPC and urinary markers significantly decline post-treatment, although after 12 months of treatment, proteinuria persisted in 5/13 (38%) dogs.

Pretreatment  $Cl_{\text{creat}}$ ,  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$  in PDH patients were above the mean values of healthy controls in 10/16 (63%), 14/19 (74%) and 11/19 (58%) dogs, respectively. Post-treatment values were 27% and 26% lower after 6 and 12 months for  $Cl_{\text{creat}}$ , 13 and 11% for  $Cl_{\text{exo}}$ , and 15% and 23% for  $Cl_{\text{endo}}$ . Between-day coefficient of variation (CV) for  $Cl_{\text{creat}}$  in our department in healthy dogs is 14.1%, for  $Cl_{\text{exo}}$  between-day CV is 9.9% and for  $Cl_{\text{endo}}$  it is 13.1%. This means that the significant decrease of  $Cl_{\text{creat}}$  at T6 and T12 and of  $Cl_{\text{endo}}$  at T12 are true changes in GFR not due to between-day measurement variability.

In the present study, 4/13 dogs had a  $Cl_{\text{creat}} < 2.0$  mL/min/kg at T12, which is compatible with renal impairment. As in previous studies, pretreatment  $Cl_{\text{creat}}$  was low and increased post-treatment, but no dog developed azotemia within 12 months.<sup>26,35</sup> Also, of the 10 dogs that died or were euthanized, none died of renal failure, although 2 dogs did have glomerular and tubulo-interstitial renal lesions at post-mortem examination. Nevertheless, it is inconclusive whether these lesions were caused by concomitant renal disease or by PDH.

In human patients with Cushing's syndrome, post-treatment GFR is decreased, leading to clinical CKD in some patients. Monitoring of GFR therefore seems mandatory in these patients.<sup>11</sup> Contrary to humans, cardiovascular risk factors do not seem to play an important role in development of renal dysfunction in dogs with Cushing's syndrome.<sup>11,36-37</sup> The clinical relevance of GFR changes in dogs with PDH is currently unclear, as in hypothyroid dogs.<sup>29,31</sup> Patients in the present study were middle-aged to old and part of the observed decline in GFR during the 12-month period could have been age-related, as described in aging humans, and suggested in aging dogs.<sup>38-39</sup>

Discrepancies between  $Cl_{\text{creat}}$ ,  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$  were observed, as previously reported in healthy dogs<sup>40</sup>, dogs with Cushing's syndrome<sup>26</sup> and healthy cats.<sup>41</sup> Potential explanations

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could be the disposition of the marker, laboratory technique, the animal's characteristics and the clinical setting. Simultaneous injection of both markers limits animal-dependent influences. Whatever the explanation, dispositions of exo- and endo-iohexol differ from each other and from creatinine so that they should be considered as different markers.

Following hypophysectomy, dogs were supplemented with cortisone acetate and levothyroxine, because of very low endogenous ACTH- and thyroid-stimulating hormone production.<sup>27</sup> Exogenous glucocorticoids<sup>42</sup> and levothyroxin<sup>29,31</sup> affect GFR, but a "physiologic dose" (0.25mg/kg bid) of cortisone was given and none of the dogs had a serum thyroxine concentration below the reference range at the time GFR was performed. Therefore, these factors are unlikely to influence GFR in the Hx group.

In agreement with a previous study,<sup>4</sup> SBP did not change significantly following treatment. At T3 and all later time points, USG was significantly higher in the Mx group, but not in the Hx group. This may be explained by impaired release of vasopressin post-hypophysectomy. All Hx dogs were supplemented with desmopressine and urine samples were taken after their morning dose.

Pretreatment UPC was increased in 68% of dogs, which is a higher percentage than in earlier studies (44 to 46%)<sup>5-6</sup>, but in accordance with more recent reports (71%).<sup>43</sup> Different cut-off values for proteinuria partly explain this difference (UPC > 1.0 in initial studies and > 0.5 in recent reports). Post-treatment proteinuria has previously been reported in 21-31% of cases with well controlled PDH and is found in 38% of dogs in the current study at T12.<sup>5-6</sup>

Because no randomization was performed, Mx and Hx groups differ at T0 and it is difficult to compare between-group post-treatment changes. In addition, both groups consist of a small number of dogs. In the Mx group, however, UPC tended to decline slower than in the Hx group. At T12 3/7 Mx dogs had an increased UPC, whereas the only 2 dogs in the Hx group with proteinuria at T6 and T12 were dogs with recurrence of PDH. These findings might suggest: 1) that recurrence of PDH is associated with recurrence of proteinuria, and 2) that surgically treated dogs may be less likely to remain proteinuric post-treatment than medically treated dogs. Therefore, proteinuria in a dog after hypophysectomy may either suggest (pending) recurrence of PDH or a concurrent disease and definitely warrants the clinician's attention.

Parallel to UPC, uALB/c, uIgG/c and uRBP/c decreased following treatment in the complete group. In a previous study comparing untreated dogs with Cushing's syndrome to healthy controls, these markers are increased, suggesting altered glomerular and tubular function.<sup>26</sup> A combined HMW (IgG) and LMW (uRBP) proteinuria indicates increased glomerular permeability with decreased tubular protein reabsorption. The latter may be due to protein overload in the tubular lumen with saturation of the tubular reabsorption capacity.<sup>44</sup> Results of the present study also indicate that these changes are reversible in most dogs. As for UPC, post-treatment course of uALB/c, uIgG/c and uRBP/c may differ in the Mx and Hx group. In the latter, uALB/c, uIgG/c and uRBP/c were lower following treatment, whereas no significant difference was found in the former. In medically treated dogs with PDH, clinical signs of hypercortisolism are controlled without removing its primary cause. Thus serum cortisol may still exceed physiological levels during a certain period of the day. Therefore, this "temporary hypercortisolism" might contribute to proteinuria in these patients, as opposed to dogs that underwent hypophysectomy and are substituted with a low dose of cortisone.

In the current study, pretreatment uNAG/c was high and decreased post-treatment, although not statistically significant. In humans with glomerulonephritis<sup>45</sup>, in geriatric cats with azotemia<sup>46</sup> and in dogs with CKD<sup>20</sup>, uNAG/c is correlated with UPC and/or albuminuria, but not with sreat concentration (or GFR in humans). Therefore, high uNAG/c may reflect high lysosomal activity rather than active tubular damage and remains high in the dogs with persistent proteinuria or albuminuria because of increased tubular processing. However, additional unknown factors also play a role, since some of the dogs with a UPC < 0.5 also have mild increases in uNAG/c.

Urinary tract infection (UTI) is known to affect UPC and may also influence urinary marker concentrations.<sup>47-48</sup> However, UTI is unlikely to affect results of the present study since only one dog had a positive urine culture prior to treatment and post-treatment and only one other dog had a cystitis at T3 and T6.

Limitations of the present study are the low number of dogs in both subgroups and not using the gold standard of urinary inulin clearance for measurement of GFR. However, this is

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a time-consuming, stressful and potentially harmful procedure, with increased risk of UTI. Moreover, both  $Cl_{\text{creat}}$  and  $Cl_{\text{iohexol}}$  show good correlation with urinary inulin clearance.<sup>16,49</sup>

In conclusion, although none of the patients developed CKD over 12 months, post-treatment decline of GFR and persistent proteinuria in 38% of dogs warrant the clinician's attention. Future research with longer follow-up and kidney histopathology of patients with persistent proteinuria or a low GFR is needed to further assess renal outcome.



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**CHAPTER VI:**  
**RENAL FUNCTION AND HISTOMORPHOLOGY IN**  
**AGED BEAGLES BEFORE AND AFTER**  
**ADMINISTRATION OF HYDROCORTISONE**

**CHAPTER VI: *Renal function and histomorphology in aged Beagles***

**RENAL FUNCTION AND MORPHOLOGY IN AGED BEAGLE DOGS BEFORE  
AND AFTER HYDROCORTISONE ADMINISTRATION**

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## **CHAPTER VI: *Renal function and histomorphology in aged Beagles***

### **Summary**

Objectives of this study were to evaluate glomerular filtration rate (GFR), renal structural changes and proteinuria in aged Beagle dogs before and after hydrocortisone administration. Eleven Beagle dogs  $\geq 10$  years old were treated with either hydrocortisone (HC group, n=6) or placebo (control group, n=5). Urinary markers, GFR and kidney biopsies were evaluated before (T0), during (T16wks) and after discontinuing HC administration (T24wks).

Results indicate that HC administration causes a significant increase in GFR. At all time points except T16wks, proteinuria was higher in the control group than in the HC group, and there was no significant difference in urinary markers between groups. At T16wks, proteinuria, urinary albumin-to-creatinine (c) ratio, immunoglobulin G/c and retinol-binding protein/c were higher compared to baseline in the HC group. At T0, rare to mild renal lesions were detected in all HC dogs and rare to moderate changes in all control dogs. Glomerulosclerosis progressed in both groups until T24wks. Tubular atrophy was detected in three HC dogs at T16wks and T24wks, but also in five control dogs throughout the study. At every time point, five HC dogs and all control dogs had rare to moderate interstitial inflammation. Rare to mild interstitial fibrosis was found in up to three HC dogs at T16wks and T24wks, and severe fibrosis in one HC dog at T24wks. Up to four control dogs had rare to mild fibrosis at all time points.

These findings indicate that clinically healthy, aged Beagle dogs may have considerable renal lesions and proteinuria, which could have implications for experimental or toxicological studies. Additional research is needed to elucidate glucocorticoid effects on renal structure, but functional changes such as hyperfiltration and proteinuria warrant attention to kidney function of canine patients with Cushing's syndrome or receiving exogenous glucocorticoids.



### **Introduction**

Proteinuria has been described in 44-75% of dogs with Cushing's syndrome and sometimes persists or even develops after successful treatment of hypercortisolism.<sup>1-2</sup> An increased urinary albumin excretion has also been demonstrated in more than 80% of human Cushing patients prior to treatment of hypercortisolism.<sup>3</sup> Whereas short-term administration of exogenous glucocorticoids (GC) seems to increase GFR in humans and dogs, one study indicates a decreased glomerular filtration rate (GFR) in people with Cushing's syndrome and suggests that renal compromise in these patients warrants more attention.<sup>4-6</sup> Therefore, human and canine Cushing patients could be considered at risk for renal complications, but information about effects of GC on renal function is scarce.

Canine endogenous hypercortisolism or Cushing's syndrome is considered a good animal model for its human counterpart, because of similarities in the pathogenesis : 80–85% of cases are caused by a pituitary tumor and ACTH-dependent, while 15–20% are ACTH-independent.<sup>7</sup> Moreover, middle-aged to old individuals are typically affected in both species and similar clinical signs include fatigue, weight gain and central obesity with muscle atrophy of the limbs, with polyuria, polydipsia and polyphagia being most pronounced in the dog.<sup>8-9</sup>

Previous experimental studies evaluating GC effects on renal function often differed from the *in vivo* situation in spontaneous Cushing's syndrome. For example, many experiments included young animals.<sup>10-11</sup> Aging itself has marked effects on renal structure and function in humans, but also in laboratory animals, dogs and cats.<sup>12-16</sup> Therefore, in aged individuals GC will act on kidneys with an already decreased renal functional reserve.<sup>17-18</sup>

In addition to serum renal markers and GFR, urinary markers are gaining interest in veterinary medicine, because of their potential for early detection of renal dysfunction and ability to localize the insult to a particular part of the nephron.<sup>19-21</sup> These markers include high molecular weight (HMW) proteins such as immunoglobulin G (IgG), intermediate molecular weight (IMW) proteins such as albumin (ALB), low molecular weight (LMW) proteins such as retinol-binding protein (RBP) and urinary enzymes such as N-acetyl- $\beta$ -D-glucosaminidase (NAG).<sup>22-23</sup> Glomerular dysfunction leads to higher filtration of ALB and in more advanced stages to presence of IgG in the ultrafiltrate.<sup>24</sup> Tubular dysfunction is reflected by urinary loss of RBP and NAG, due to disturbed reabsorption or increased protein trafficking and structural damage of tubule cells, respectively.<sup>25-26</sup>

## **CHAPTER VI: *Renal function and histomorphology in aged Beagles***

Information about GC effects on renal function and morphology is rare, and often limited to case reports, despite their frequent therapeutic use and the occurrence of Cushing's syndrome in humans and dogs.<sup>11,27-30</sup> Therefore, the objectives of this study were to evaluate GFR, renal light microscopic and ultrastructural changes, and proteinuria in old Beagle dogs before and after administration of hydrocortisone.

### **Material and methods**

#### ***Animals***

This study was approved by the Ethical Committee of Ghent University (EC: 2008/146). Eleven female spayed Beagle dogs, age 8.4 to 11 years (median: 10 years), with a body weight ranging from 10.9 to 14 kg (median 12.9 kg) were included in the study. Dogs were housed in pairs in two adjacent indoor kennels of 2.6 m<sup>2</sup> each, with free access to water, fed a standard dry food<sup>a</sup> (25.0% protein, 15.3% fat, 52.0% carbohydrates, 2.6% fiber) twice a day, and allowed outdoor activity in a secured area. They were judged healthy based on physical examination, hematology, biochemistry profile, abdominal ultrasound and two consecutive morning urinary corticoid-to-creatinine ratio's ( $< 10^{-6}$ ) to exclude endogenous Cushing's syndrome. A urinalysis consisting of sediment and dipstick analysis, urine specific gravity (USG), urinary protein-to-creatinine ratio (UPC), and bacterial culture were performed, but proteinuria (UPC > 0.5) was not an exclusion criterion.

#### ***Study design***

Five dogs were randomly assigned to the placebo group (control group) and six to the hydrocortisone treatment group (HC group). After baseline sampling (T0), the control group received a gelatin capsule containing lactose and the HC-group received a capsule containing lactose and a median dose of 9.6 mg/kg HC (range from 8.7 to 11.0 mg/kg) every 12 hours (h). Hydrocortisone was used because it is the synthetic glucocorticoid most closely resembling endogenous cortisol and dosage was based on two previous publications.<sup>10-11</sup> Dogs were monitored for development of clinical signs of GC excess such as polyuria, polydipsia and polyphagia, and for skin abnormalities using a scoring sheet. Treatment period was 16 weeks (end of treatment period: T16wks), then the HC dose was tapered over 4 weeks (one week 10 mg/kg once a day, two weeks 5 mg/kg once a day, and one week 2.5 mg/kg once a day, end of tapering period: T20wks) and the study ended four weeks after completely stopping treatment (end of study: T24wks). Plasma exogenous creatinine-iohexol clearance tests (PEC-ICT), kidney biopsies and urine sampling were performed in both groups at baseline, at T16wks and at T24wks. Blood and urine samples were additionally collected during treatment after 4 wks (T4wks) and at T20wks.

At T16wks and T24wks, an ACTH-stimulation test was performed in all dogs by collecting serum for measurement of cortisol before and 60 minutes (min) after intramuscular injection of 0.25 mg of synthetic ACTH<sup>b</sup>. Cortisol concentrations were measured using an immunoassay<sup>c</sup> validated for use in the dog.

## CHAPTER VI: *Renal function and histomorphology in aged Beagles*

### *Sampling methods*

Morning urine samples were taken by cystocentesis (10 mL, 22 G needle). Urinary dipstick analysis<sup>d</sup>, urine specific gravity (USG), urinary protein-to-creatinine ratio (UPC), sediment analysis and bacterial culture were performed. After centrifugation, the urine supernatant was stored at  $-80^{\circ}\text{C}$  until analysis of urinary markers.

### *Plasma exogenous creatinine-iohexol clearance test (PEC-ICT)*

All dogs were fasted for at least 10 h prior to the test day and fed immediately after ending the sampling period. Water was provided *ad libitum*. GFR was measured by plasma clearance of exogenous creatinine ( $\text{Cl}_{\text{creat}}$ ), exo- and endo-iohexol ( $\text{Cl}_{\text{exo}}$  and  $\text{Cl}_{\text{endo}}$ ), adapted from a protocol previously proposed in cats and described in Chapter V.<sup>31-32</sup>

Plasma creatinine concentrations were analysed using an enzymatic method<sup>f</sup>. This technique was validated by measuring samples with increasing creatinine concentration 4 times per day, on each of three consecutive days. The upper limit of quantification was 1202  $\mu\text{mol/L}$ . Within- and between-day coefficients of variation were  $< 3\%$  in the lower, middle, and high concentration range (80, 530 and 1160  $\mu\text{mol/L}$ , respectively), and there was linear correlation between theoretical and measured concentrations within quantification limits. The basal plasma creatinine concentration measured on the day of PEC-ICT testing was subtracted from the creatinine concentrations measured in the samples from that dog.

Plasma concentrations of iohexol stereo-isomers (exo- and endo-iohexol) were determined by a validated high-performance liquid chromatographic method with ultraviolet detection, as described in previous studies and in Chapter V.<sup>33</sup> Pharmacokinetic analyses were performed using WinNonlin<sup>g</sup>. For clearance calculation, individual plasma data were subjected to noncompartmental analysis, as described by Watson and coworkers.<sup>34</sup> Plasma  $\text{Cl}_{\text{creat}}$ ,  $\text{Cl}_{\text{exo}}$  and  $\text{Cl}_{\text{endo}}$  were determined as mentioned in Chapter V.

### *Serum and urinary renal markers*

Serum creatinine ( $\text{screat}$ )<sup>f</sup> and urea<sup>h</sup> were measured using an enzymatic method. Urinary protein was determined with a turbidimetric method using benzethonium chloride<sup>h</sup> and urinary creatinine with a modified Jaffé reaction using picric acid<sup>g</sup>. As markers of glomerular function, uALB and uIgG concentrations were determined with a commercial canine specific enzyme-linked immunosorbent assay (ELISA)<sup>i</sup>. As an indication of tubular function, uRBP was determined with a human ELISA<sup>i</sup> and uNAG activity using a colorimetric assay<sup>j</sup>. All assays have been previously described and validated in our

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laboratory.<sup>22,35</sup> Within- and between-day coefficients of variation were 5.2 and 12.0% for the uALB ELISA, 6.1 and 7.7% for the uIgG ELISA, 4.3 and 5.5% for the uRBP ELISA, and 4.9 and 8.1% for the uNAG colorimetric assay. Because spot urine samples were used, urinary concentration of each marker was indexed to urinary creatinine (c) and expressed as a ratio: uALB/c, uIgG/c, uRBP/c and uNAG/c.<sup>36,37</sup>

### ***Kidney biopsies***

Coagulation was checked in all dogs by measurement of buccal mucosal bleeding time, and activated partial thromboplastin and prothrombin time<sup>k</sup>. Ultrasound guided percutaneous kidney biopsies were taken under general anesthesia at T0, T16wks and T24wks. Dogs were premedicated intravenously (IV) with a combination of acepromazine<sup>l</sup> (0.01 mg/kg) and butorphanol<sup>m</sup> (0.01 mg/kg). Afterwards, dogs received IV diazepam<sup>n</sup> (0.2 mg/kg) immediately followed by propofol<sup>o</sup> to effect until endotracheal intubation could be performed. Anesthesia was maintained with isoflurane<sup>p</sup> (2% vaporized in 100% oxygen).

Ultrasound guided kidney biopsies were taken by an experienced radiologist, according to a previously described technique.<sup>38</sup> Large-bore 14-gauge needles<sup>q</sup> with 9cm length and 20 mm specimen notch were used to take two consecutive biopsies > 10 mm long (or three biopsies < 10 mm long) from the left kidney. Biopsy cores were collected and transported according to a standardized protocol adapted from Lees and co-workers.<sup>39</sup> Specimens were transversely divided in three parts and transferred into a standard container with 3% glutaraldehyde for EM within 5 min after collection, in 10% buffered formalin for LM and in Michel's transport medium for IF. Biopsies were cooled and transported to a nephropathology service within 24 h.

Three  $\mu\text{m}$  sections from each biopsy sample were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Masson's trichrome and periodic acid methenamine silver. All biochemical stains were performed according to standard procedures.<sup>39</sup> Slides were evaluated using light microscopy for presence of glomerular, tubular, interstitial and vascular lesions, using a tie-grade system from 1 to 4 (1=rare lesion, 2=mild, 3=moderate, 4=severe).

For direct immunofluorescence (IF) examination, fresh unfixed renal specimens were embedded in OCT compound, snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Subsequently, five  $\mu\text{m}$  thick sections were fixed with acetone for 15 min. After washing with PBS (two passages), slides were incubated with FITC-labelled sheep anti-dog IgG<sup>r</sup>, goat anti-dog IgM<sup>r</sup>, goat anti-canine IgA<sup>r</sup>, goat anti-dog C3<sup>r</sup>, rabbit anti-human  $\kappa$  light chain<sup>s</sup>, rabbit anti-human  $\lambda$  light chain<sup>s</sup>, and rabbit anti-human complement factor C1Q<sup>s</sup>. Primary antibodies

## **CHAPTER VI: *Renal function and histomorphology in aged Beagles***

were omitted as negative controls and substituted with PBS.<sup>40</sup> Fluorescence findings were classified according to the pattern seen on immunofluorescent examination (granular or linear), which included the localization of the deposit (mesangium, GBM, tubules and vessels), the distribution (focal, diffuse, segmental and global) and the intensity.

For transmission electron microscopy (EM), the tissue was post-fixed in 2% osmium (in distilled water) for 1.5 h, dehydrated in graded acetone and embedded in epon. Semi-thin sections were stained with azure methylene blue. Ultrathin sections were counterstained with uranyl acetate and lead citrate. For ultrastructural studies, a Philips EM 420 was used. All histological sections were examined in a blinded fashion by three pathologists.

### ***Statistical analysis***

Analyses were performed with a commercial software program<sup>†</sup>. Level of significance was set at 5%. A general linear model was used to test the effect of HC treatment, time point and their interaction between the control and the HC group. When a statistically significant interaction between treatment and time point was detected, a post hoc hypothesis test was performed to compare the two groups at each period.

### **Results**

#### ***Dogs***

All dogs assigned to the HC group developed clinical signs of hypercortisolism, such as polyuria, polydipsia and skin changes (atrophy of abdominal skin with phlebectasia, no regrowth of clipped haircoat, comedones, ecchymose) and complementary laboratory changes (increased alkaline phosphatase and alanine aminotransferase, ALT). Additionally, some dogs also developed muscle atrophy and abdominal distention, but no significant changes in bodyweight were detected throughout the study. At T16wks, 36 h after HC withdrawal, all dogs in the HC group failed to respond adequately to ACTH-stimulation; median (range) serum cortisol concentrations before and after ACTH-injection were 18 nmol/L (6-30 nmol/L) and 25 nmol/L (17-86 nmol/L). At T16wks serum ALT was significantly increased in the HC group compared to the control group ( $p < 0.001$ ).

At T24wks, 4 weeks after complete HC withdrawal, clinical signs were resolved, except for skin changes in some dogs, and laboratory changes returned to baseline (alkaline phosphatase and ALT within reference interval). Response to an ACTH-stimulation test was within normal range in all dogs; median (range) serum cortisol concentrations before and after ACTH-injection were 32 nmol/L (25-60 nmol/L) and 330 nmol/L (246-516 nmol/L).

#### ***Plasma exogenous creatinine-iohexol clearance test***

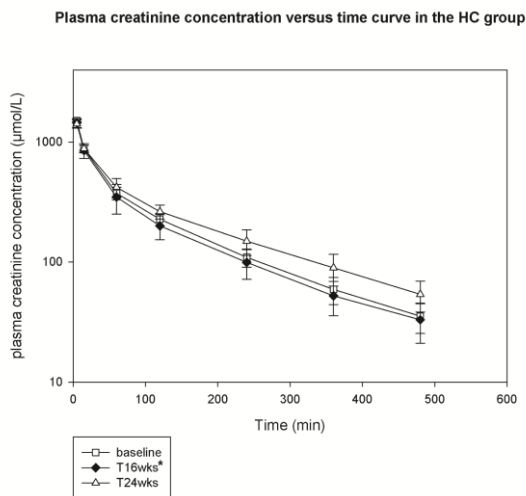
As presented in Table 1, plasma  $Cl_{\text{creat}}$  and  $Cl_{\text{exo}}$  were significantly higher in the HC group than in the control group at T16wks ( $p < 0.001$ ). There was no significant difference between groups either at baseline or at T24wks. Plasma  $Cl_{\text{endo}}$  was not significantly different between groups at any time point ( $p=0.21$ ). Figure 1 shows the plasma creatinine (A), exo-iohexol (B) and endo-iohexol concentration (C) versus time curves in the control and HC groups at baseline, T16wks and T24wks.

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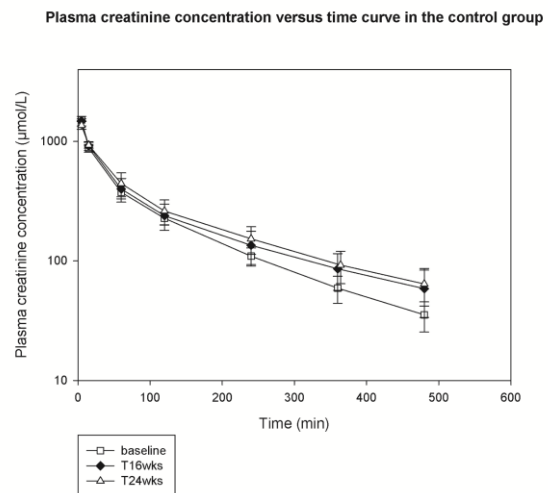
**Figure 1. Plasma concentration versus time curves.**

Plasma creatinine (A1, A2), exo-iohexol (B1, B2) and endo-iohexol concentration (C1, C2) versus time curves in the hydrocortisone (HC) group (6 dogs) and the control group (5 dogs) at T0, T16wks and T24wks. Mean  $\pm$  standard deviation is displayed. The asterisk indicates a statistically significant difference ( $p < 0.001$ ) in plasma clearance between groups at T16wks, for creatinine and exo-iohexol clearance.

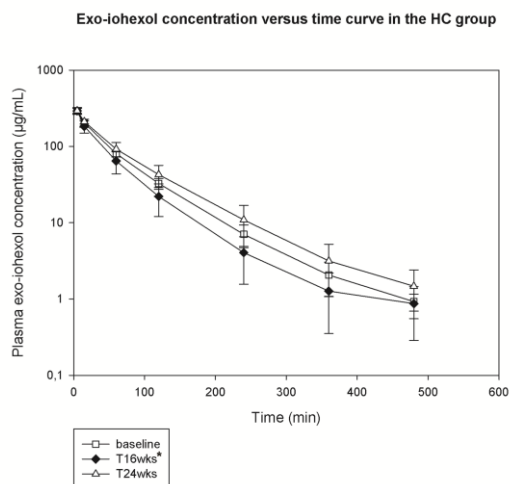
**A1**



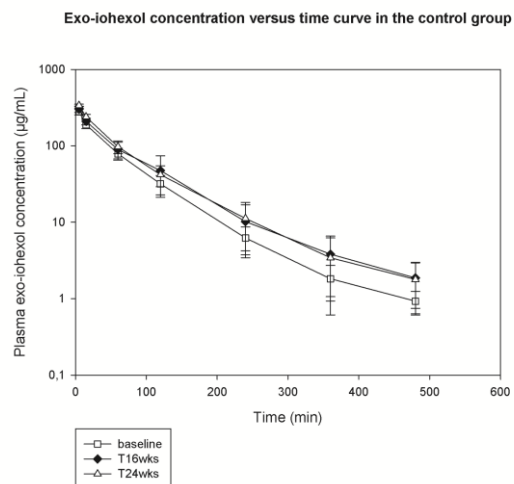
**A2**



**B1**



**B2**



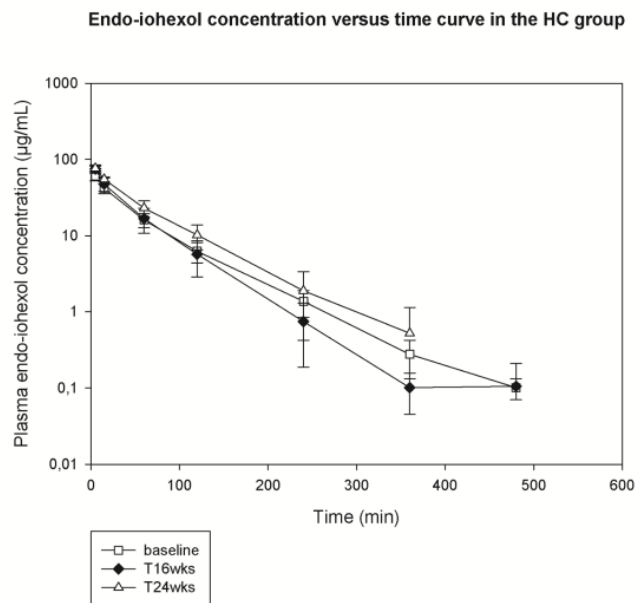


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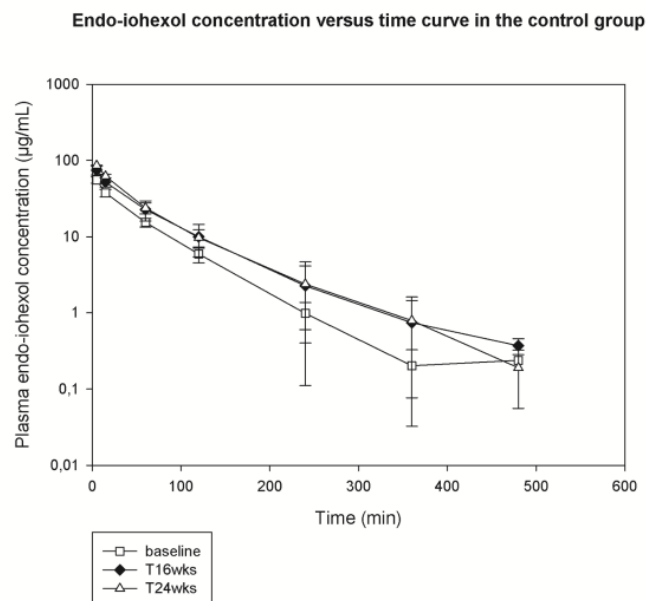
**Figure 1 (continued). Plasma concentration versus time curves.**

Plasma creatinine (A1, A2), exo-iohexol (B1, B2) and endo-iohexol concentration (C1, C2) versus time curves in the hydrocortisone (HC) group (6 dogs) and the control group (5 dogs) at T0, T16wks and T24wks. Mean  $\pm$  standard deviation is displayed. The asterisk indicates a statistically significant difference ( $p < 0.001$ ) in plasma clearance between groups at T16wks, for creatinine and exo-iohexol clearance.

**C1**



**C2**



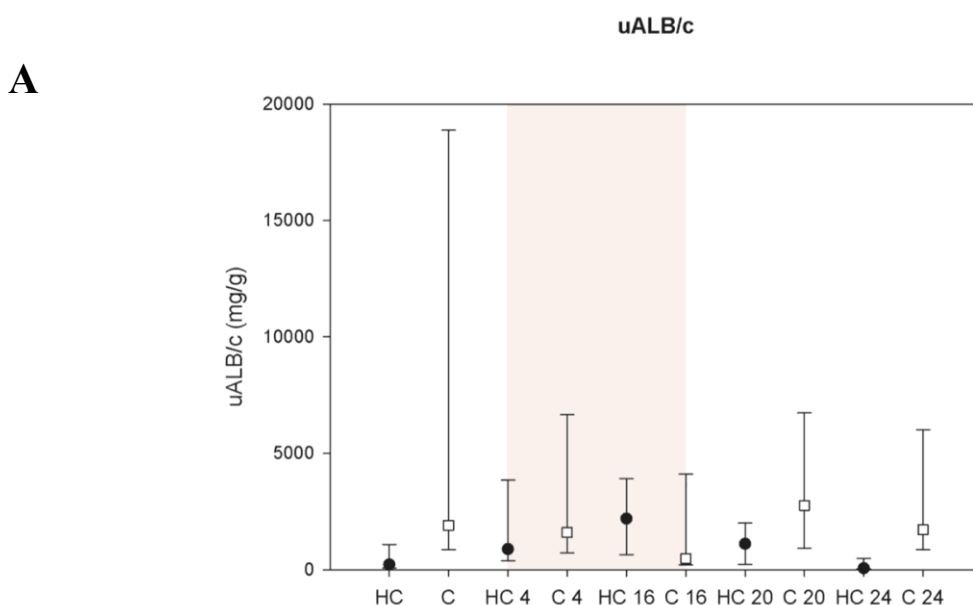
## CHAPTER VI: Renal function and histomorphology in aged Beagles

### Serum and urinary renal markers

As shown in Table 1, no significant differences were detected between groups for screat ( $p = 0.74$ ), urea concentration ( $p = 0.32$ ) and USG ( $p = 0.30$ ) throughout the study, despite a decrease of USG during treatment in the HC group. UPC was significantly higher in the control group than in the HC group ( $p = 0.003$ ) at baseline, T4wks, T20wks and T24wks, but not at T16wks. Figure 2 presents the median and 75-25<sup>th</sup> percentiles of UPC, uALB/c, uIgG/c, uRBP/c and uNAG/c in the HC and control groups before, during and after HC administration, with the grey area representing the treatment period. Differences between HC and control groups in glomerular markers uALB/c ( $p = 0.05$ ) and uIgG/c ( $p = 0.26$ ) did not reach statistical significance, although they progressively increased within the HC group during treatment and decreased afterwards. Also for tubular markers uRBP/c ( $p = 0.07$ ) and uNAG/c ( $p = 0.20$ ), there was no significant difference between the HC and control groups at any time point, despite the fact that uRBP/c seemed to increase within the HC group, with highest concentrations at T16wks, and declined again after discontinuation of HC.

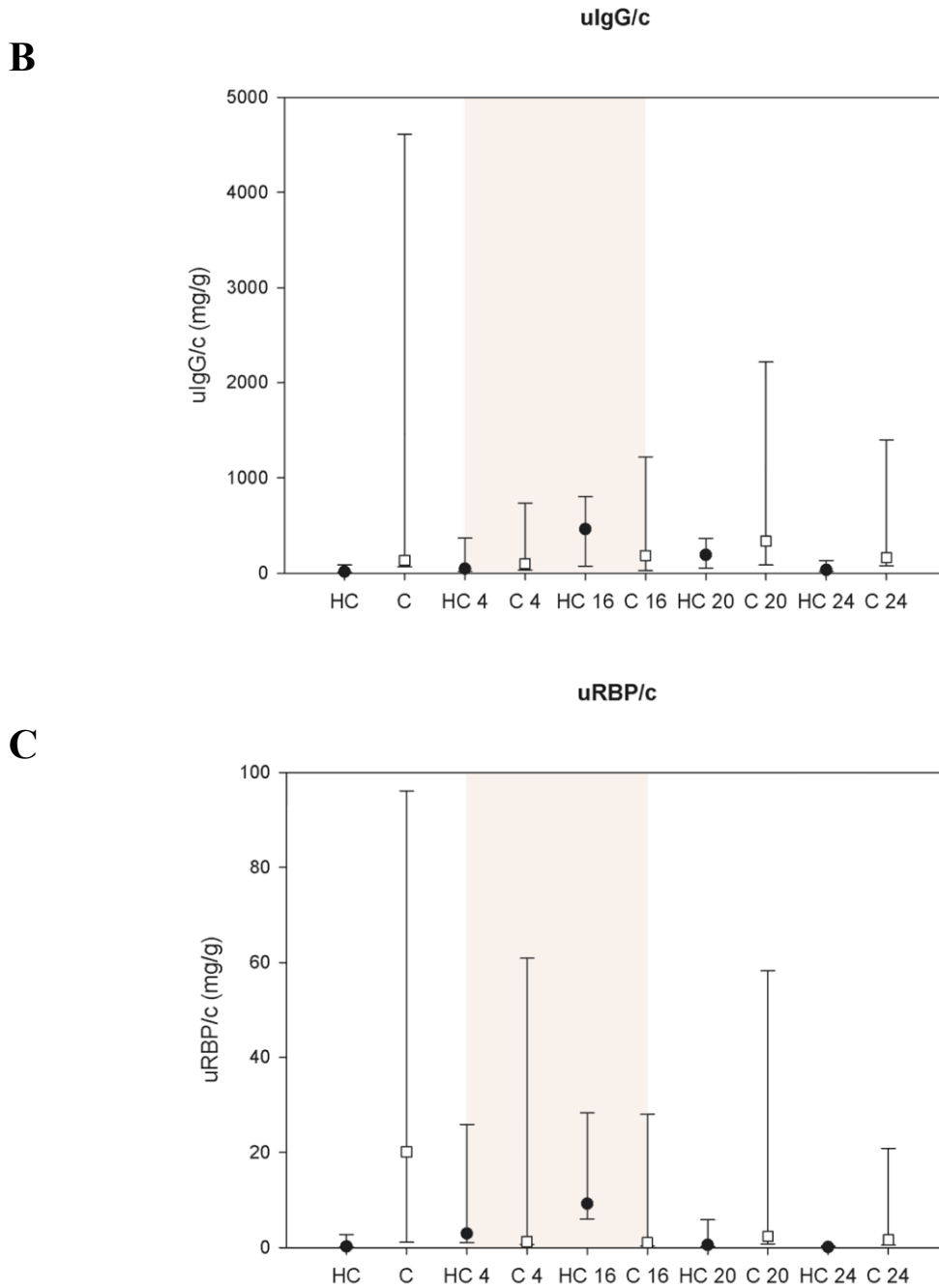
#### Figure 2. Urinary markers.

Median and 75-25<sup>th</sup> percentiles of urinary albumin-to-creatinine ratio (uALB/c) (A), immunoglobulin G-to-creatinine ratio (uIgG/c) (B), retinol-binding protein-to-creatinine ratio (uRBP/c) (C) and N-acetyl- $\beta$ -D-glucosaminidase ratio (uNAG/c) (D) at T0, T16wks and T24wks in the hydrocortisone (HC) group (black circles) and in the control (C) group (white squares). The grey area indicates the hydrocortisone administration period.



**Figure 2 (continued). Urinary markers.**

Median and 75-25<sup>th</sup> percentiles of urinary albumin-to-creatinine ratio (uALB/c) (A), immunoglobulin G-to-creatinine ratio (uIgG/c) (B), retinol-binding protein-to-creatinine ratio (uRBP/c) (C) and N-acetyl- $\beta$ -D-glucosaminidase ratio (uNAG/c) (D) at T0, T16wks and T24wks in the hydrocortisone (HC) group (black circles) and in the control (C) group (white squares). The grey area indicates the hydrocortisone administration period.

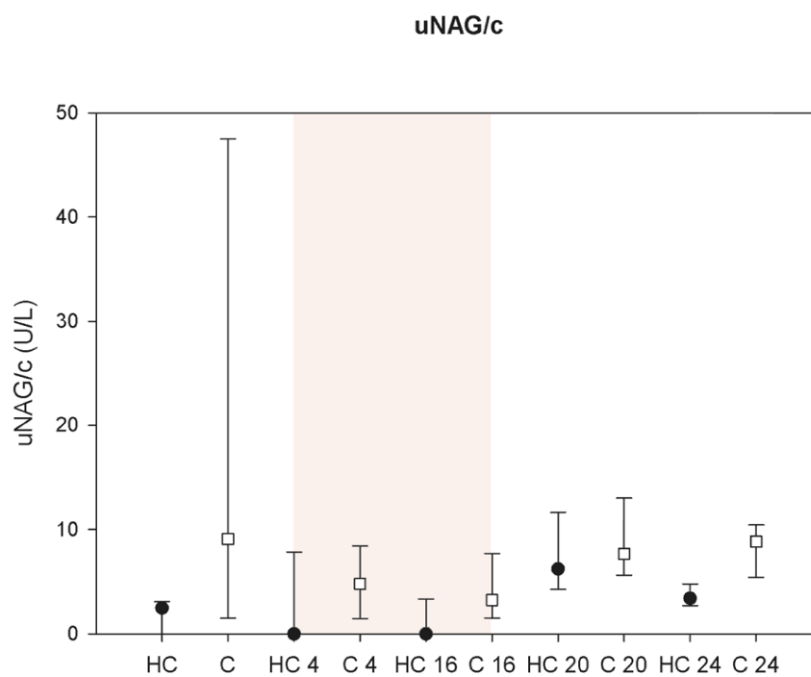


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### Figure 2 (continued). Urinary markers.

Median and 75-25<sup>th</sup> percentiles of urinary albumin-to-creatinine ratio (uALB/c) (A), immunoglobulin G-to-creatinine ratio (uIgG/c) (B), retinol-binding protein-to-creatinine ratio (uRBP/c) (C) and N-acetyl- $\beta$ -D-glucosaminidase ratio (uNAG/c) (D) at T0, T16wks and T24wks in the hydrocortisone (HC) group (black circles) and in the control (C) group (white squares). The grey area indicates the hydrocortisone administration period.

**D**



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**Table 1. Glomerular filtration rate and routine renal markers in the control and HC group.**

T0, baseline; T16wks, at the end of the 16 weeks hydrocortisone treatment period; T24 wks, at 4 weeks after complete cessation of hydrocortisone treatment;  $Cl_{creat}$ , plasma clearance of creatinine;  $Cl_{exo}$ , plasma clearance of exo-iohexol,  $Cl_{endo}$ , plasma clearance of endoiohexol; screat, serum creatinine, USG urinary specific gravity; UPC, urinary protein-to-creatinine ratio. The asterisk indicates in which group results are significantly increased ( $p < 0.05$ ) compared to the other group at that timepoint.

Renal Marker	T0		T16wks		T24wks	
	Control	HC	Control	HC	Control	HC
$Cl_{creat}$ (mL/min/kg)	3.3 (2.8-3.7)	3.3 (2.6-3.7)	3.0 (2.1-3.4)	3.7 (2.5-4.2)*	2.8 (2.0-3.0)	2.7 (2.3-3.3)
$Cl_{exo}$ (mL/min/kg)	2.9 (2.7-3.6)	3.2 (2.5-3.7)	2.9 (2.1-3.4)	4.0 (2.5-4.5)*	2.9 (2.1-3.1)	2.8 (2.0-3.3)
$Cl_{endo}$ (mL/min/kg)	3.1 (2.8-3.9)	2.9 (2.3-4.5)	2.5 (1.6-2.9)	3.2 (1.8-3.9)	2.3 (1.8-2.4)	2.3 (1.6-2.7)
screat ( $\mu$ mol/L)	72 (58-92)	80 (75-84)	86 (75-113)	88 (68-111)	90 (76-119)	90 (68-107)
urea (mmol/L)	13.1 (7.3-17.2)	11.0 (9.9-14.6)	10.3 (8.1-25.9)	9.2 (6.0-20.4)	11.8 (7.7-27.4)	10.8 (8.1-16.3)
USG	1.018 (1.008-1.035)	1.024 (1.012-1.050)	1.018 (1.011-1.020)	1.003 (1.000-1.013)	1.012 (1.006-1.050)	1.019 (1.007-1.029)
UPC	1.77 (0.12-10)*	0.18 (0.13-1.56)	1.93 (0.11-6.55)	3.04 (0.28-6.19)	1.95 (0.15-6.67)*	0.16 (0.02-0.33)

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### *Kidney histopathology*

Most important light microscopic findings in HC and control dogs are presented in Table 2 and 3. Affected dogs in both groups and the score ranges of severity at different time points are mentioned.

Commonly observed glomerular lesions were global endocapillary and mesangial hypercellularity, with increase of mesangial matrix. Increase of mesangial matrix progressed in both the HC and control group at T16wks and T24wks, with an increasing number of affected dogs and highest scores at T24wks (Figure 3a, 3b and 3c). Obsolescent glomeruli were detected in one control dog at T0, in 4 HC dogs and all control dogs at T16wks, and in all dogs at T24wks (Figure 4a). Asymmetrical thickening and splitting of Bowman's capsule was most pronounced in two control dogs especially at T24wks, whereas only rare to mild changes were seen in the HC group, mainly at T16wks (Figure 4b). Tubular lesions included tubular atrophy, tubular macro- and micro-vesiculation and cellular pigment. In the HC group there was no tubular atrophy at T0, whereas mild to severe atrophy was detected in three dogs at T16wks and T24wks. Rare to moderate tubular atrophy was present throughout the study in three to five control dogs. At every time point, up to five HC dogs and all control dogs had rare to moderate interstitial inflammation (Figure 3d). Rare to mild interstitial fibrosis was found in up to three HC dogs at T16wks and T24wks, and severe fibrosis in one HC dog at T24wks. Up to four control dogs had rare to mild fibrosis at all time points.

Ultrastructural lesions included changes of the glomerular basement membrane (GBM) such as thickening, rarefaction, wrinkling and irregular jagged profile (Figure 5a). At T0, EM detected GBM changes in only 2/6 HC dogs, but they were prominent in the entire HC group at T16wks and T24wks. In the control group mild to moderate GBM lesions were found at all time points. Multifocal to diffuse podocyte foot process fusion was found in 5/6 HC dogs and all five control dogs at every time point (Figure 5b and 5c). Degenerative tubular cells and/or degenerative changes in podocytes, endothelial and mesangial cells were detected in dogs from both groups and were most pronounced at T24wks.

Immunofluorescence staining was unremarkable with coarse, segmental deposits of Ig G (1+ to 2+) in the capillary wall in 1/6 HC dogs and 2/5 control dogs at T16wks. Mesangial Ig M deposits (1+ to 2+) were detected in 4/6 HC dogs and 3/5 control dogs at T16wks, and in one control dog at T24wks. This was probably a nonspecific finding due to entrapment of Ig M in sclerotic glomeruli. Coarse  $\lambda$  light chain deposits (1+ to 2+) were seen in the capillary walls and mesangium of one dog from each group at T16wks, and in one control dog at

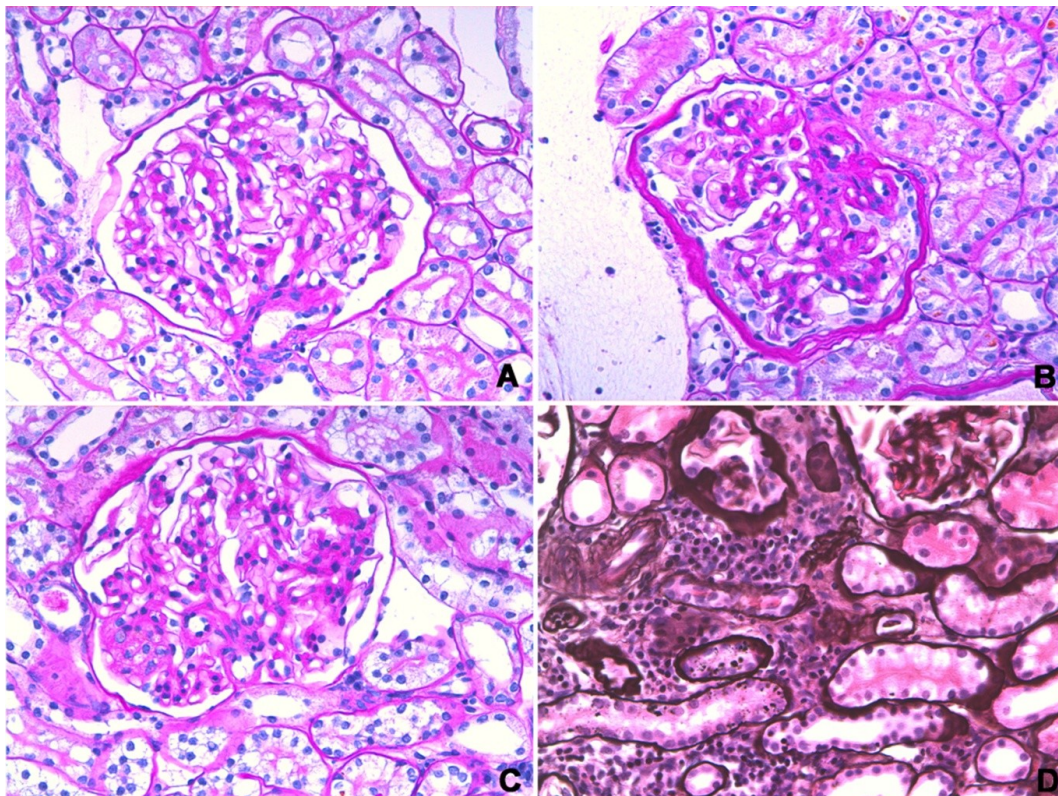
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T24wks. One dog from the HC group had 1+ coarse, diffuse, segmental deposits of C3 at T24wks.

In summary, glomerular, tubular and interstitial lesions presented in Table 2 and 3 progressed over time in both groups, with most severe lesions detected at T24wks. At T16wks, a marked increase was seen in the number of HC dogs with obsolescent glomeruli and tubular atrophy compared to baseline. No significant vascular changes (hypertrophic and hyaline arteriosclerosis, arteritis, fibrinoid necrosis, thrombosis) were present at baseline or at any of the other time points.

### **Figure 3. Light microscopic lesions.**

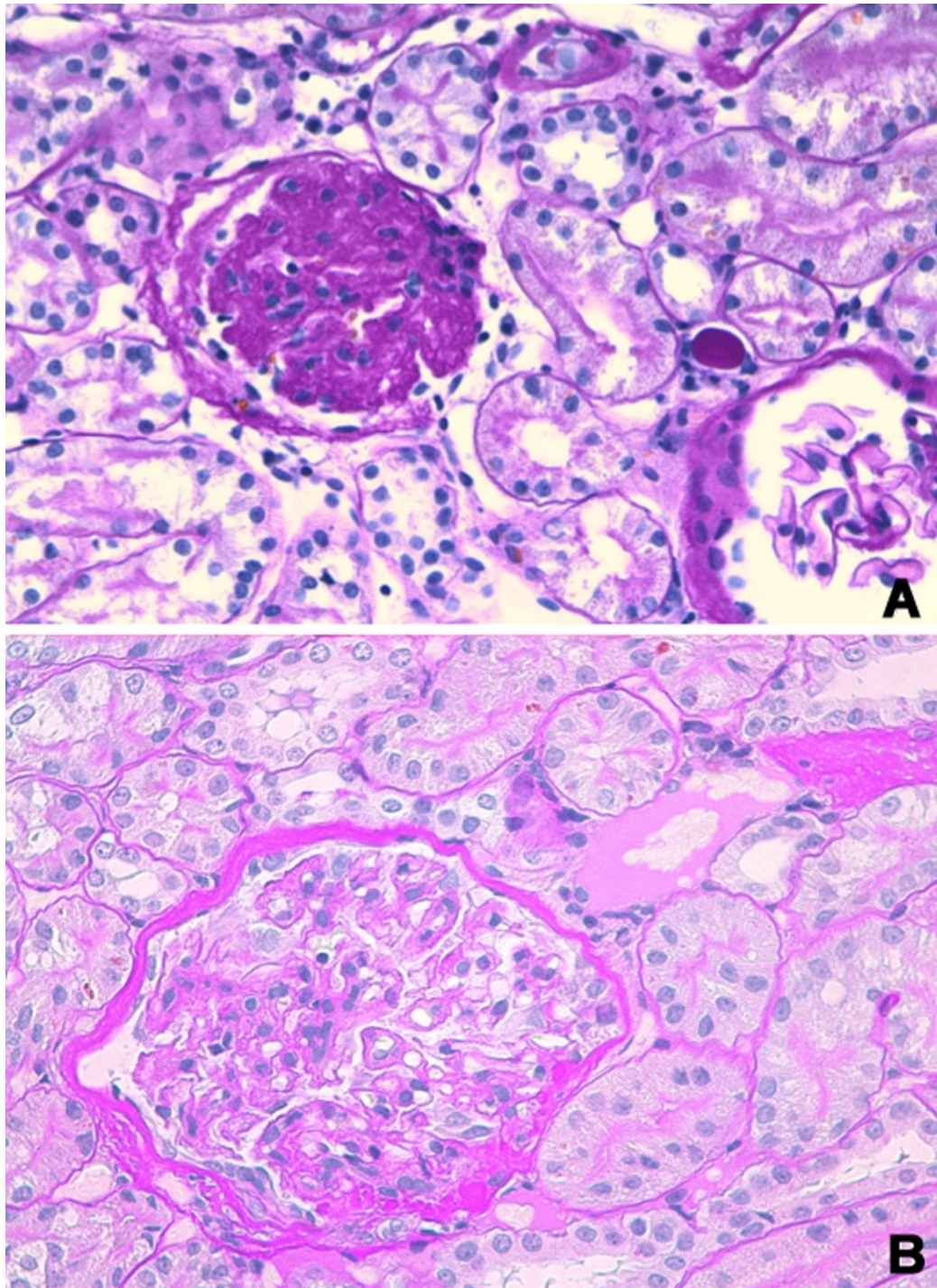
(A) Glomerulus, Dog 11 at T0. Early glomerular lesions including minimal increase of mesangial matrix. Periodic acid-Schiff staining (x400); (B) Glomerulus, Dog 11 at T16 weeks. Moderate hypercellularity of mesangial and endothelial cells and one synechia. There is segmental to global increase of mesangial matrix and Bowman's capsule is symmetrically thickened with proliferation of parietal epithelial cells. Periodic acid-Schiff staining (x400); (C) Glomerulus, Dog 11 at T24 weeks. Moderate hypercellularity of mesangial and endothelial cells associated with global increase of mesangial matrix. Visceral epithelial cells are hypertrophic. Periodic acid-Schiff staining (x400); (D) Tubulo-interstitium, Dog 11 at T24 weeks. Moderate inflammation widely separating tubules and atrophic tubules. Periodic acid-methenamine silver staining (x400).



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**Figure 4. Glomerular lesions.**

(A) Glomerulus, Dog 8 at T16 weeks. Obsolescent glomerulus. Periodic acid-Schiff staining (x400).  
(B) Glomerulus, Dog 5 at T24 weeks. Accentuated lobulation, increase of mesangial and endothelial cellularity of the tuft, and synechia. Splitting of Bowman's capsule is visible. Periodic acid-Schiff staining (x400).

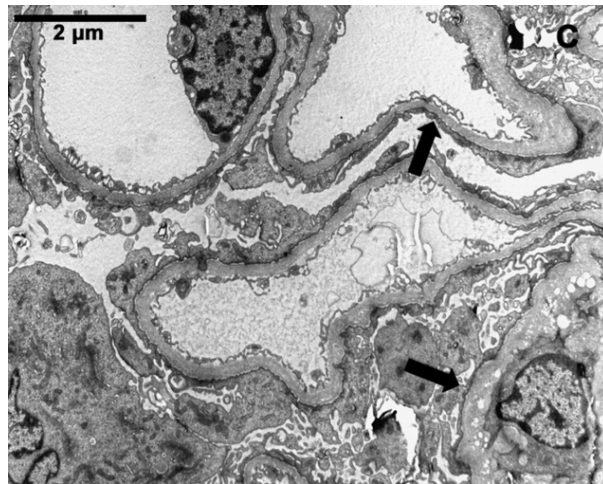
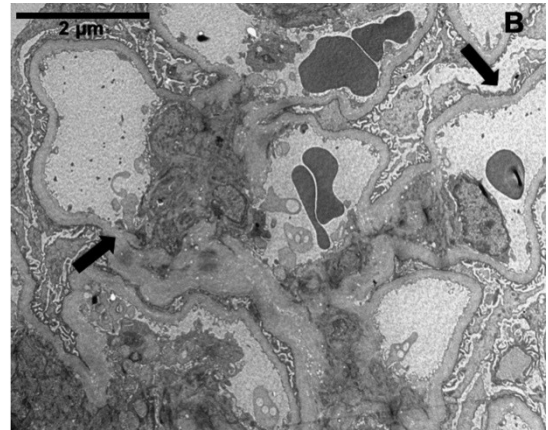
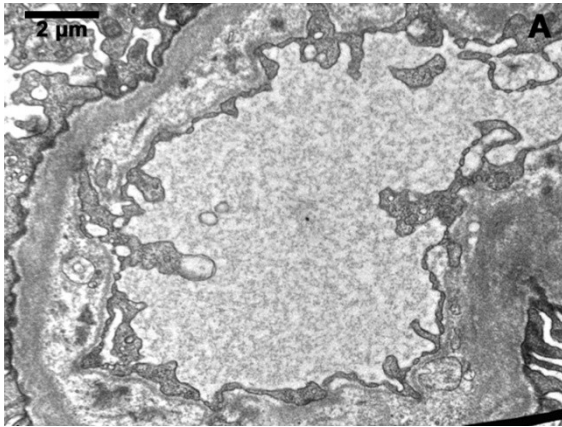




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### Figure 5. Electron microscopic glomerular lesions.

(A) Glomerulus, Dog 10 at T16 weeks. Sub-endothelial widening and rarefaction of the glomerular basement membrane associated with foot process effacement. EM x5000; (B) Glomerulus, Dog 9 at T24 weeks. Multifocal foot process effacement (arrow) and endothelial cell swelling with reduction of the capillary lumens. Increased mesangial matrix. EM x4000; (C) Glomerulus, Dog 11 at T24 weeks. Diffuse and severe foot process effacement (arrow). EM x400.



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**Table 2. Glomerular lesions in the hydrocortisone and control group.**

Endocap., endocapillary; Prim.mesang., primary mesangial; T0, baseline; T16wks, at the end of the 16 weeks hydrocortisone treatment period; T24 wks, at 4 weeks after complete cessation of hydrocortisone treatment; HC, hydrocortisone. Score: 1=rare lesion, 2=mild, 3=moderate, 4=severe.

Dog number	Global endocap. hypercellularity			Prim. mesang. hypercellularity			Diffuse glomerulosclerosis			Global glomerulosclerosis			Number of obsolescent glomeruli		
	T0	T16 wks	T24 wks	T0	T16 wks	T24 wks	T0	T16 wks	T24 wks	T0	T16 wks	T24 wks	T0	T16 wks	T24 wks
<b>HC group</b>															
Dog 1	0	0	2	0	0	2	2	2	4	2	3	2	0	5/94	5/39
Dog 2	0	0	0	0	0	2	0	0	4	0	0	2	0	0	4/63
Dog 3	2	2	2	2	3	2	0	2	4	0	2	3	0	3/55	9/90
Dog 4	0	0	0	0	0	0	1	1	3	1	1	2	0	3/52	2/26
Dog 5	0	0	0	0	2	1	2	2	3	2	2	2	0	4/56	4/35
Dog 6	0	0	2	0	0	2	2	2	3	2	0	2	0	0	4/37
<b>Control group</b>															
Dog 7	2	2	2	3	3	2	3	3	4	3	3	2	0	6/44	6/83
Dog 8	0	2	2	0	2	2	2	4	3	2	4	2	5/12	18/79	4/19
Dog 9	0	0	0	0	0	0	0	0	2	0	0	2	1/22	3/47	5/42
Dog 10	3	2	2	2	3	3	3	2	2	3	2	2	6/39	8/57	4/46
Dog 11	0	0	0	0	0	0	0	2	2	0	1	1	0	2/69	1/45

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**Table 3. Tubular and interstitial lesions in the hydrocortisone and control group.**

T0, baseline; T16wks, at the end of the 16 weeks hydrocortisone treatment period; T24 wks, at 4 weeks after complete cessation of hydrocortisone treatment; HC, hydrocortisone. Score: 1=rare lesion, 2=mild, 3=moderate, 4=severe.

Dog number	Tubular atrophy			Tubular cell pigment			Interstitial fibrosis			Interstitial inflammation		
	T0	T16wks	T24wks	T0	T16wks	T24wks	T0	T16wks	T24wks	T0	T16wks	T24wks
<b>HC group</b>												
Dog 1	0	3	2	2	2	2	0	2	2	0	2	2
Dog 2	0	0	0	1	1	1	0	0	2	1	0	2
Dog 3	0	2	4	1	1	1	0	1	4	1	2	3
Dog 4	0	3	3	2	2	2	0	0	2	0	2	2
Dog 5	0	0	0	2	2	2	0	0	0	0	1	0
Dog 6	0	0	0	1	0	2	0	0	0	0	0	1
<b>Control group</b>												
Dog 7	1	2	0	0	2	1	0	2	2	1	3	2
Dog 8	3	3	2	2	2	2	2	2	2	2	3	2
Dog 9	0	2	2	0	0	2	0	2	2	1	2	2
Dog 10	2	2	2	2	2	2	2	0	2	2	2	2
Dog 11	0	2	0	2	2	2	0	2	0	1	2	2

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### **Discussion**

This study demonstrates that HC administration reversibly increases GFR in aged Beagle dogs. Plasma  $Cl_{\text{creat}}$  and  $Cl_{\text{exo}}$  were both significantly increased in the HC group at T16wks, but no longer differed between groups eight weeks after tapering and cessation of treatment. This is in agreement with previous reports showing that synthetic GC augment GFR in dogs, with an increase in renal plasma flow (RPF), renal vasodilation and a decrease in filtration fraction (FF).<sup>5,41</sup> The mechanism for this increase is most likely at least partly species-specific, since GC also increase GFR in humans, but with little effect on RPF and an increase instead of a decrease in FF.<sup>42</sup> Suggested mechanisms include hemodynamic factors, interaction of GC with vaso-active hormones and catabolic effects leading to an increase in plasma amino acids, which in turn augments RPF and GFR.<sup>43,44</sup> Glucocorticoids can also mediate GFR changes through an increased blood pressure, leading to a higher intraglomerular pressure, although they do not always cause hypertension in dogs.<sup>10</sup> However, blood pressure measurements were not available in the current study.

Although all dogs included in the study were clinically healthy with no abnormalities on hematology, biochemistry profile and abdominal ultrasound, two dogs from the HC group and four dogs from the control group had an increased UPC at baseline. Proteinuria, which did not progress over a two year follow-up period, has been previously described in otherwise healthy Beagles by Stuart and coworkers.<sup>45</sup> In the latter study, LM and EM changes were similar to our results and degree of proteinuria correlated with the severity of glomerular lesions.<sup>45</sup> In the present study, dogs with most severe glomerular changes also had the highest UPC (n° 3: 1.56, n° 7: 1.77, n° 8: 10 and n° 10: 6.4; reference range < 0.5). The dog with the highest UPC had severe glomerulosclerosis, was the only dog with obsolescent glomeruli at baseline and had the highest percentage of obsolescent glomeruli throughout the study. Pre-existing renal lesions and proteinuria, independent of GC administration, in these healthy aged Beagle dogs raise questions for interpretation of similar findings in dogs with glomerular diseases. Moreover, this important observation has to be kept in mind when using Beagle dogs as a model for human renal disease or toxicological studies.<sup>46</sup>

Proteinuria was significantly higher in the control group than in the HC group at all time points, except for T16wks. This could be caused by an increase in UPC in the HC group at the end of the treatment period. Table 1 and Figure 2 indicate that UPC was increased in the HC group at T16wks and progressively decreased again at T20wks and T24wks. Figure 2 also suggests an increase followed by a decline within the HC group for uALB/c, uIgG/c and

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uRBP/c, but not for uNAG/c. Nevertheless, dogs with the highest UPC also had the highest uNAG/c, which could be an indication of increased tubular lysosomal protein degradation. Interestingly, changes in the lysosomal degradation pattern with altered distribution of lysosomes from the perinuclear to the apical tubular cell region were also detected in proteinuric X-linked Alport Syndrome affected dogs.<sup>24</sup>

Possible mechanisms causing glomerular proteinuria include glomerular hypertension and structural alterations of the glomerular barrier.<sup>47</sup> A GC-induced increase in GFR together with hemodynamic alterations could augment glomerular pressure and contribute to proteinuria. This hypothesis is supported by the parallel increase of both GFR and proteinuria at the end of the HC treatment period and their simultaneous decline after cessation of treatment in the HC group. Structural alterations that may contribute to proteinuria were glomerulosclerosis, podocyte foot process effacement, and GBM changes such as wrinkling, thickening, delamination and rarefaction. Whereas proteinuria and hyperfiltration were rapidly reversible after cessation of treatment in the HC group, the structural changes seemed to persist at T24wks. Possible explanations for this are three-fold: 1) the lesions are not related to GC administration, but are age-dependent and continue their physiological progression over time, 2) the lesions are GC-induced and there is a longer lag-time than eight weeks between tapering and cessation of HC administration and normalization of structural changes, and 3) the lesions are irreversible. Because it was not possible to repeat kidney biopsies at a later time in the current Beagle colony, the present study cannot confirm or dispute any of these hypotheses.

At baseline, many dogs had mild to moderate pre-existing renal lesions. Glomerulosclerosis has been described to be age-related in dogs and in people.<sup>46,48</sup> Tubular atrophy, and interstitial inflammation and fibrosis have also been shown to increase with aging in both species.<sup>46,49,50</sup> The brownish cellular pigment detected throughout the study in many dogs is most likely lipofuscin accumulation, which again may be an age-related phenomenon as previously suggested in rats and dogs.<sup>49,51</sup> Interestingly, no vascular morphological changes were found throughout the current study period in any of the groups, whereas in people vascular changes are part of age-related renal lesions.<sup>52,53</sup>

At the end of the HC administration period, most striking LM findings in the HC group, not detected at baseline, were obsolescent glomeruli and tubular atrophy. However, there was a higher prevalence of renal lesions at baseline than expected and glomerular,

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tubular and interstitial changes also progressed in the control group. Therefore, it is not possible to separate HC effects from progression of pre-existing renal lesions. It is possible that HC administration accelerated age-related glomerulosclerotic and tubular changes in the HC group, but definitive conclusions cannot be drawn from these data. Another limitation of the study is the lack of IF examination at baseline. However, IF findings at T16wks and T24wks were either mild or non-specific.

In conclusion, this study shows that HC administration reversibly increases GFR, with a similar trend for proteinuria. Results indicate that clinically healthy aged Beagle dogs have renal lesions and proteinuria, which could have implications for experimental or toxicological studies. Although further research is needed to elucidate whether morphological renal changes are GC-induced, hyperfiltration and proteinuria are and warrant attention to kidney function in human and canine patients with Cushing's syndrome or receiving exogenous GC.

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### **Sources and manufacturers**

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- p. Vet-Core<sup>TM</sup>, Surgivet, USA
- q. Bethyl Laboratories Inc., Montgomery, TX, USA
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**CHAPTER VII:**  
**GENERAL DISCUSSION**

## CHAPTER VII: *General discussion*

Fifty-five to 80% of human and up to 86% of canine patients with Cushing's syndrome have systemic hypertension, and about 40% of the cases are resistant to treatment of hypercortisolism.<sup>1-5</sup> In addition, 44 to 75% of affected dogs have proteinuria, which also persists post-treatment in around 30% of cases.<sup>4,6</sup> Systemic hypertension can cause target-organ damage to the kidney and contribute to renal proteinuria.<sup>7</sup> Proteinuria is a sign of renal dysfunction, but may also play a role in progression of kidney disease.<sup>8,9</sup>

Consequently, dogs with Cushing's syndrome could be considered at risk for renal complications. Current knowledge on the link between GCs and kidney function is reviewed in Chapter I. Surprisingly, information on renal function in human and canine patients with Cushing's syndrome was found to be scarce. Only two studies in the nineties described hypertension and proteinuria in dogs with Cushing's syndrome. However, their cut-off for proteinuria was higher than the one currently defined, further long-term follow-up post-treatment was not available and dogs were treated with mitotane, while nowadays trilostane is used for medical treatment.<sup>4,6</sup>

Screat and urea are known to be insensitive markers of renal function, hence the need for markers that allow early detection of renal dysfunction. Glomerular filtration rate is considered the best overall index of renal function. In human medicine, some urinary markers might allow early and site-specific indication of renal dysfunction.<sup>10</sup>

Therefore, the main objective of this thesis was to evaluate renal function in dogs with Cushing's syndrome using routine renal markers as well as GFR and urinary markers.

### **1. Measurement of urinary markers in dogs**

At the start of this research, only few studies had described measurement of uALB, uRBP and uNAG in veterinary medicine.<sup>11-17</sup> Moreover, uIgG and uCRP had never been measured in canine patients. Therefore a solid assay validation was mandatory, as well as more information about urine sampling procedures and sample storage conditions for these "new" urinary markers.

In a first study, we evaluated effects of sampling method and storage temperature (-20° compared to -80°) on concentrations of uALB, uRBP and uNAG (Chapter III).

To avoid lower urinary tract contamination, urinary samples for bacterial culture are generally taken in a sterile manner by cystocentesis in dogs and cats.<sup>18</sup> Midstream voided samples are contaminated with lower urinary tract epithelial cells, proteases and bacteria, but have the advantage they can easily be collected by the owner at home. We collected morning

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urine samples by cystocentesis and midstream free catch in client-owned dogs, healthy as well as diseased, and found no significant difference in urinary marker concentrations in cystocentesis compared to voided samples. Therefore, analysis of urinary markers generally does not seem to be influenced by sample type. Caution is still warranted, because one dog had markedly elevated uALB, uRBP and uNAG in the voided sample. Urine culture was negative, but it was hypothesized that preputial disease or urethral inflammation caused contamination of the voided sample that may have affected the urinary markers.

It is still unknown whether urinary tract inflammation or infection (UTI) affects urinary marker concentrations. Experimental cystitis and urinary blood contamination have been shown to increase UPC in dogs and therefore guidelines advise to exclude UTI before evaluating the source of proteinuria.<sup>7,19</sup> In this first study, we conducted a pilot experiment to assess effects of *in vitro* hematuria, pyuria, and bacteriuria on urinary marker concentrations. Results suggested minimal effects of experimental hematuria, pyuria and bacteriuria on uALB, uNAG, and uRBP concentrations. However, these findings have to be interpreted with caution, because *in vitro* addition of leucocytes or bacteria to urine does not fully reflect an *in vivo* inflammatory process. To date, there are no studies evaluating urinary marker concentrations in dogs during and after resolution of UTI.

Our study was the first to investigate effects of storage temperature and time on urinary marker concentration in canine samples. In conclusion, samples for analysis of uALB and uRBP can be stored at  $-20^{\circ}\text{C}$  for up to 4 months, whereas  $-80^{\circ}\text{C}$  is preferred for storage up to one year. Urinary NAG enzymatic activity is less stable at  $-20^{\circ}\text{C}$  as well as  $-80^{\circ}\text{C}$ , with already substantial losses after 4 months at  $-20^{\circ}\text{C}$  and a more severe decline after one year at  $-80^{\circ}\text{C}$ .

This first study (Chapter III) provided us with important practical information for our subsequent research. In the following studies we decided to collect urine samples by cystocentesis, to be able to perform bacterial cultures, and all urine samples were stored at  $-80^{\circ}\text{C}$ .

In the second study (Chapter IV), the first objective was to validate a canine ALB ELISA and a uNAG colorimetric test. Secondly, we wanted to compare two glomerular markers, uALB and uCRP, and two tubular markers, uRBP and uNAG between healthy and CKD dogs. A last objective was to detect a possible age-related difference in these markers between healthy young and older dogs.

Assay characteristics (limit of detection, within- and between day-variability, and linearity after serial dilution) of the uALB ELISA and uNAG colorimetric test were all satisfactory. Validation of ELISA kits for measurement of uIgG, uCRP and uRBP was performed in cooperation with the Laboratory of Biochemistry and is described elsewhere by Maddens and coworkers.<sup>20</sup>

Urinary ALB/c, uRBP/c and uNAG/c were significantly higher in dogs with CKD than in healthy controls. These results indicate that combined use of glomerular and tubular markers in conjunction with traditional tests may provide more detailed information about the extent and location of renal damage. Urinary markers appear to be promising as non-invasive tools for the diagnosis of CKD in dogs. An important question is how “early” can urinary markers detect a decreased renal function, in other words, how sensitive are they? One recent study has attempted to answer this question by investigating uALB/c and uRBP/c in healthy dogs and dogs with different stages of CKD, separated into five groups based on screat, UPC and GFR.<sup>21</sup> Urinary ALB and uRBP could not differentiate between healthy control dogs and nonazotemic, nonproteinuric dogs with reduced  $Cl_{creat}$ . Therefore, the authors concluded that uALB/c and uRBP/c were not diagnostically useful in detecting a mildly decreased  $Cl_{creat}$  at an early stage of renal disease. However, GFR measurement and  $Cl_{creat}$  reference values in the former study can be questioned. Plasma  $Cl_{creat}$  was measured using a limited sampling technique (3 samples) “during the period from 4 to 9 hours after injection”. When using a limited sampling strategy, choice of blood sampling times is critical.<sup>22</sup> For plasma  $Cl_{creat}$  it has been shown that sampling within the first hour after injection is required to minimize considerable errors in GFR calculation.<sup>23</sup> Furthermore, in dogs with CKD, samples at a later time than 9 hours post-injection may be required for accurate GFR calculation.<sup>23</sup> The lower limit of normal for  $Cl_{creat}$  was based on values obtained in six Beagle dogs, that were only six months old.<sup>24</sup> It has been shown that GFR in two month old Beagle puppies is higher than that in adult dogs, so it is warranted to use an age-matched control group when defining “normal range”  $Cl_{creat}$ .<sup>25</sup> Consequently, further research in dogs classified according to stage of renal disease is necessary to investigate the urinary markers’ ability for early detection of CKD.

In this study (Chapter IV), no significant difference was found between healthy dogs and dogs with CKD for uCRP/c. C-reactive protein is an important positive acute phase protein in dogs and differs from the other urinary markers. For CRP to appear in urine, its plasma concentration must be increased and the glomerular barrier must be sufficiently damaged to allow HMW protein filtration. None of the healthy dogs had any detectable uCRP

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and only three of the CKD dogs did. Thus, one possible hypothesis is that the increased uCRP/c in these CKD dogs reflects an inflammatory response leading to increased plasma concentrations and subsequent leakage of CRP through the damaged glomerular barrier. In humans, inflammation and oxidative stress start early in the process of failing kidney function and mild increases in CRP concentration are present even in patients with moderate renal impairment.<sup>26,27</sup> In dogs with CKD, however, increases in serum CRP seem to be variable, as is the case in dogs with pyometra.<sup>28</sup> Because of this variability and the fact that circulating levels of CRP are expected to be low in dogs with cortisol excess, we decided to measure uIgG instead of uCRP as a HMW glomerular marker in our subsequent studies.<sup>29</sup>

Regarding the last aim of this study, there was no difference in urinary markers between healthy dogs of 1-3 years old and healthy dogs  $\geq 7$  years old. Although the number of dogs was too small to define actual reference ranges for the urinary markers, the study in Chapter IV provided essential information on assay validation, urinary marker concentrations in healthy dogs and in dogs with kidney disease. Because Cushing's syndrome occurs in middle-aged to old dogs, the absence of an age-related difference in the urinary markers was a relevant finding especially for our patient group.

### **2. Renal function in dogs with Cushing's syndrome**

In Chapter V we used measurement of routine renal markers, GFR and urinary markers to evaluate our literature-based hypothesis that Cushing's syndrome may affect renal function in dogs.

In a first cross-sectional study (Chapter V, § 5.1), screat and USG were lower, while UPC, uALB/c, uIgG/c, uRBP/c and uNAG/c were significantly higher in the dogs with Cushing's syndrome than in the healthy controls. Plasma  $Cl_{\text{endo}}$  but not  $Cl_{\text{exo}}$  was also higher in the affected dogs. Urea and SBP were not different between groups. One dog with ATH and one dog with PDH had concomitant CKD. After exclusion of these two dogs, the overall results remained similar.

Regarding routine renal variables, results were similar to those reported by other authors. Screat was decreased by about 30% in the Cushing group, as previously reported.<sup>30</sup> However, screat concentration is not only affected by renal function, but also by the production of creatinine from creatine-phosphate in skeletal muscle, as shown in dogs with experimental CKD and hypothyroidism.<sup>23,31,32</sup> In Cushing patients, both decreased muscle mass and increased GFR may contribute to a lower screat. Urea is usually within or in the



lower part of the reference range in these patients.<sup>33</sup>

In our study, median UPC was somewhat higher than previously reported, with the highest UPC found in a dog with combined PDH and CKD (UPC 16.8), although one dog with only ATH and two dogs with PDH also had very high UPC's (10.2, 10.1 and 16.3, respectively), in absence of UTI.<sup>4,6,34</sup> Unique to this study is that it demonstrated proteinuria in dogs with Cushing's syndrome to have a combined glomerular and tubular component, illustrated by HMW (uIgG) and LMW (uRBP) protein loss. Glomerular filtration of plasma proteins may result from glomerular hypertension or altered glomerular permselectivity. The latter mechanism may certainly play a role, since the glomerular barrier is normally impermeable to IgG, which was highly increased in the dogs with Cushing's syndrome.<sup>35</sup> Tubulo-interstitial injury leads to impairment of megalin-mediated endocytosis by proximal tubular cells and an increased urinary RBP.<sup>36</sup> Similarly, tubular cell dysfunction leads to increased leakage of lysosomal enzymes such as uNAG into the urine.<sup>37</sup>

This study was the first to evaluate GFR in dogs with Cushing's syndrome. We found that plasma  $Cl_{\text{endo}}$  but not  $Cl_{\text{exo}}$  was increased in the affected dogs compared to healthy control dogs. Differences in plasma clearance of the two stereo-isomers have previously been described and indicate that dispositions of exo- and endo-iohexol may vary in different clinical settings and that they need to be viewed as different markers.<sup>38</sup> Exo-iohexol is the major stereo-isomer and is typically considered a marker of GFR in dogs.<sup>39</sup> Short term administration of GCs increases GFR in humans, dogs and other species.<sup>40-42</sup> In contrast, a decreased GFR was observed in human patients with Cushing's syndrome.<sup>43</sup> Therefore, GFR may be higher in dogs with short duration of disease and lower in dogs with longstanding Cushing's syndrome, leveling out differences in  $Cl_{\text{exo}}$  between affected and healthy dogs in our study.

This study also showed that glomerular and tubular function is altered in untreated dogs with Cushing's syndrome. The discrepancy between  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$  did not allow us to draw definitive conclusions regarding GFR. The next step in our research was therefore to conduct a longitudinal study to determine the effect of treatment on these changes and to better investigate long term variations of GFR in Cushing patients.

The second study in Chapter V (§ 5.2) therefore aimed to longitudinally assess renal function in dogs with PDH (Cushing's *disease*) before and after treatment with trilostane (Mx group) or transsphenoidal hypophysectomy (Hx group).

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In this study, UPC, uALB/c, uIgG/c and uRBP/c significantly declined following treatment of hypercortisolism in the complete group of dogs, indicating that hypercortisolism causes reversible proteinuria in most dogs, similar to results in previous studies.<sup>6,44</sup> However, it is important to note that 1) proteinuria persisted in almost one third of the dogs and 2) post-treatment decrease of proteinuria seemed to be more pronounced in surgically treated than in medically treated dogs. In the Mx group, decline of UPC was slower and at T12 3/7 dogs had an increased UPC. In the Hx group, UPC was already significantly decreased at T1 and moreover, the only two dogs with a mild proteinuria at T6 and T12 were dogs with recurrence of Cushing's disease. These findings suggest that 1) recurrence of PDH is associated with recurrence of proteinuria, and 2) surgically treated dogs may be less prone to post-treatment proteinuria than medically treated dogs. However, further research is warranted to confirm the latter results because of the lack of randomisation and the small number of dogs in each group.

In the Hx group, uALB/c, uIgG/c and uRBP/c were lower following treatment, whereas no significant difference was found in the Mx group. In medically treated dogs with PDH, clinical signs of hypercortisolism are controlled without removing the primary cause of the disease. Thus, serum cortisol may still exceed physiological levels during a certain period of the day. Therefore, it is tempting to hypothesize that this "temporary hypercortisolism" might contribute to proteinuria in these patients, as opposed to dogs that underwent hypophysectomy and are substituted with physiological doses of cortisone.

There was no significant change in uNAG/c ratio post-treatment. In humans with glomerulonephritis<sup>45</sup>, in geriatric cats with azotemia<sup>46</sup> and in dogs with CKD<sup>47</sup>, it has been demonstrated that uNAG/c is correlated with UPC and/or albuminuria, but not with creatinine concentration (or GFR in humans). Therefore, increased uNAG/c may reflect high lysosomal activity rather than active tubular damage. In our study, post-treatment uNAG/c may remain high in the dogs with persistent proteinuria or albuminuria because of increased tubular protein processing.

As in § 5.1, results indicate a combined HMW (uIgG) and LMW (uRBP) proteinuria in dogs with Cushing's disease. Exactly how GC excess enhances glomerular filtration of protein is currently unknown. Hemodynamic as well as structural alterations of the glomerular barrier may play a role.<sup>44</sup>

In our follow-up study, GFR was high pre-treatment and declined post-treatment, paralleled by a decrease in proteinuria and urinary markers. Therefore, a cortisol-induced increase in GFR may increase glomerular pressure and contribute to proteinuria.

It would have been very interesting to examine kidney biopsies pre- and post-treatment for evaluation of structural changes, especially in patients with persistent proteinuria. However, this would cause the study protocol to be even more intensive, leading to owners declining participation of their pet. Post-mortem kidney histopathology was available for two dogs. The first dog had quite prominent glomerular and tubular lesions, despite never being proteinuric during the study. The second dog had mild glomerular lesions and focal interstitial inflammation and was only proteinuric at baseline. Renal histopathology was not standardized, and electron microscopic and immunofluorescent examinations were not available. No baseline kidney biopsies were taken, so these lesions may have been pre-existing and independent of the Cushing's disease.

Regarding GFR,  $Cl_{\text{creat}}$  and  $Cl_{\text{endo}}$  were significantly decreased post-treatment in the complete group. None of the dogs developed azotemia during the follow-up period or died of renal-related causes, but GFR was  $< 2$  ml/min/kg in four dogs at the end of the study, a value previously suggested to be compatible with renal impairment.<sup>32,48,49</sup> In human patients with Cushing's syndrome, post-treatment GFR was also shown to be decreased, with four of 18 patients being identified with CKD. The authors concluded that monitoring of GFR seems mandatory in these patients and that the low GFR may have consequences for individual drug dosage.<sup>43</sup> However, cardiovascular risk factors and atherosclerosis may play an important role in the pathophysiology of renal consequences in humans with Cushing's syndrome, whereas in dogs these factors seem less important.<sup>50,51</sup> The clinical relevance of GFR changes in dogs with Cushing's disease is currently unclear, as is the case for the observed GFR decrease in hypothyroid dogs.<sup>32,48</sup> Dogs included in the current study were middle-aged to old and part of the observed decline in GFR during the 12-month period may have been age-related. Glomerular hypofiltration is a well known phenomenon in aging humans, and one study suggested a decrease of  $Cl_{\text{creat}}$  in aging dogs.<sup>52,53</sup> Effects of Cushing's disease on GFR might have clinical implications in patients with concurrent renal or cardiac dysfunction, which is not uncommon in a geriatric population. Both are associated with a low GFR, which might be masked by the hypercortisolism-induced increase in GFR.<sup>49</sup> Whether these patients could be at risk for deterioration of renal function after treatment of Cushing's disease is currently unknown.

The studies in chapter V show that glomerular and tubular function is altered in dogs with Cushing's syndrome, causing an increased GFR and a combined HMW and LMW proteinuria. There is a post-treatment decline in GFR, which was not associated with

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development of CKD during the one year follow-up period. Proteinuria is reversed after treatment in most dogs, but persisted in 43% of dogs treated with trilostane and in two dogs with recurrence of PDH after hypophysectomy, but not in any of the other surgically treated dogs.

### **3. Renal function and morphology in Beagle dogs before and after hydrocortisone administration**

Because the study in § 5.2 did not include renal biopsies, it remained unclear whether cortisol-excess could also cause structural renal changes. Furthermore, it remained to be examined if the observed functional changes in GFR and protein excretion were reproducible in an experimental setting. Although effects of exogenous GC on renal function had been studied in dogs, this was never done in older dogs, nor using GFR, urinary markers and standardized kidney histopathology including both light and electron microscopy.<sup>44,54</sup>

Therefore, objectives of the following study (Chapter VI) were to evaluate GFR, renal histological and ultrastructural changes, and proteinuria in old Beagle dogs before and after administration of hydrocortisone (HC).

In this study, GFR was increased after 16 weeks of HC administration and decreased to baseline values after tapering and cessation of the treatment, which corroborates our previous findings in dogs with spontaneous Cushing's syndrome (Chapter V). A possible mechanism for this increase in GFR is GC-interaction with the renin-angiotension-aldosterone system (RAAS). In dogs with Cushing's disease, plasma renin activity and aldosterone concentration are low and in dogs treated with dexamethasone, intrarenal angiotensin II and angiotensin converting enzyme-activity is decreased, which leads to renal vasodilation.<sup>55,56</sup> In combination with an increased cardiac output and renal blood flow as discussed in Chapter I, this causes an increased GFR. Following treatment with trilostane, plasma renin activity is increased, which may lead to RAAS-activation and renal vasoconstriction.<sup>56,57</sup> Renal vasoconstriction causes contraction of mesangial cells, which lowers available filtration surface in the glomerulus and decreases the ultrafiltration coefficient.<sup>58</sup> Together with a decreased circulating volume and renal blood flow, GFR declines after cessation of HC administration or after treatment of Cushing patients with trilostane.

As in both studies in Chapter V there was a discrepancy between  $Cl_{\text{exo}}$  and  $Cl_{\text{endo}}$ . Nevertheless, in these healthy dogs,  $Cl_{\text{exo}}$  and not  $Cl_{\text{endo}}$  was significantly increased, in contradiction to our previous studies in patients with Cushing's syndrome. One hypothesis might therefore be that the two stereo-isomers behave differently according to disease status.

Although all dogs included in the study were clinically healthy with no abnormalities on CBC, biochemistry profile and abdominal ultrasound, two dogs from the HC group and four dogs from the control group had unexpectedly high UPCs at baseline. The dog with the highest UPC had severe glomerulosclerosis, obsolescent glomeruli at baseline and the highest percentage of obsolescent glomeruli throughout the study. Pre-existing renal lesions and proteinuria, independent of HC administration, in these healthy aged Beagle dogs raise questions for interpretation of similar findings in dogs with glomerular diseases. Moreover, this important observation has to be kept in mind when using Beagle dogs as a model for human renal disease or toxicological studies.<sup>59</sup>

Renal lesions found at baseline may have been age-related. Glomerulosclerosis has been described to be age-related in dogs and in people.<sup>59,60</sup> Tubular atrophy, interstitial inflammation and fibrosis have also been shown to increase with aging in both species.<sup>61,62</sup> Throughout the study, brownish cellular pigment was detected in many dogs, which is most likely lipofuscin accumulation and again may be an age-related phenomenon, previously documented in rats and dogs.<sup>61,63</sup>

At the end of the HC administration period, most striking light microscopic findings in the treatment group, not detected at baseline, were obsolescent glomeruli and tubular atrophy. However, there was a higher prevalence of renal lesions at baseline than expected and glomerular, tubular and interstitial changes also progressed in the control group. Therefore, it is not possible to separate HC effects from progression of pre-existing renal lesions. It is possible that HC administration accelerated age-related glomerulosclerotic and tubular changes in the HC group, but definitive conclusions cannot be drawn from these data.

Proteinuria was significantly higher in the control group than in the HC group at all time points, except for T16wks. This can be explained by an increase of UPC in the treatment group at T16wks, followed by a decrease after tapering and cessation of HC treatment. No significant changes between both groups were detected for the urinary markers. However, similar to UPC, uALB/c, uIgG and uRBP/c tended to increase during HC treatment and declined afterwards within the HC group. These findings again suggest a reversible increase in proteinuria, similar to our findings in previous studies (Chapter V). Proteinuria and hyperfiltration were rapidly reversible after cessation of treatment in the HC group, supporting a link between hemodynamic glomerular changes and proteinuria.

Interestingly, the longitudinal study in Chapter V (§5.2) and the study in Chapter VI both showed different results for uNAG/c in comparison to the other markers. Although uNAG/c is increased in untreated dogs with Cushing's syndrome compared to healthy controls

## **CHAPTER VII: *General discussion***

(Chapter V, §5.1), its post-treatment decline was not statistically significant, as opposed to the other urinary markers (Chapter V, §5.2). Time course of uNAG/c during administration of HC (Chapter VI) did not show an increasing trend, followed by a decline after cessation of HC treatment, as documented for the other urinary markers (Chapter VI, Figure 2).

Urinary NAG is a lysosomal enzyme with several isoenzymes, and in case of tubular impairment not only total uNAG will increase but also isoenzyme ratios will change.<sup>64</sup> Our assay only measures total uNAG and it would be very interesting to measure the isoenzymes to better characterize the changes in uNAG activity. Furthermore, the NAG assay is based on a colorimetric technique, which may be prone to more analytical interaction than the antibody-based ELISAs for measurement of uALB, uIgG and uRBP.

### **4. Future perspectives**

Our results show that urinary markers are increased in dogs with CKD as well as in dogs with Cushing's syndrome, that do not have azotemia. These data suggest that the urinary markers may be sensitive indicators of early renal dysfunction, in contrast to routine renal markers. However, we did not investigate their ability to detect mild decreases in GFR. Raila and coworkers (2010) reported that uALB and uRBP cannot distinguish dogs with a normal GFR from those with a mildly decreased GFR.<sup>21</sup> Even if this suggestion is confirmed in future studies, urinary markers may still be useful to monitor response to therapy or predict a uremic crisis in dogs or cats with CKD. Consequently, further research in patients with different stages and etiologies of renal disease is necessary.

We have not tested the specificity of the urinary markers and therefore future studies should assess to which extent urinary markers are influenced by concurrent diseases. In our studies, concurrent systemic disease was an exclusion criterion.

The use of urinary markers is limited by the relatively expensive and labour-intensive assays needed for their analysis. If future studies prove them to be useful clinical tools, simplified analytical methods should be developed to allow use in clinical settings. The same is true for measurement of GFR. A sophisticated HPLC-UV method was used to simultaneously determine exo- and endo-iohexol, whereas plasma Cr can be measured with much simpler techniques. Plasma clearance of exogenous Cr is therefore the most feasible method to measure GFR in practice, but an exogenous Cr solution is not commercially available and limited sampling strategies need to be further optimised.<sup>23</sup>

Human and canine patients with Cushing's syndrome are predisposed to thromboembolic disease.<sup>66-68</sup> We did not test coagulation and it would be very interesting to

investigate this in Cushing patients with and without proteinuria, since hypercoagulability has been associated with urinary loss of anti-thrombin III, among other mechanisms.<sup>33,65</sup>

Regarding proteinuria in general, many questions remain unanswered. Follow-up of a large number of Cushing patients with post-treatment proteinuria over a longer time period, including kidney biopsies may provide more in-depth information. In human and canine patients with CKD, hypertension and proteinuria are associated with progression of disease and proteinuria may contribute to tubulo-interstitial damage.<sup>9,68,69</sup> Therefore, ACE-inhibitors (and in humans also angiotensin receptor-blockers) are widely used as anti-hypertensive and anti-proteinuric agents to slow progression of renal disease in both species.<sup>70,71</sup> It remains to be investigated whether Cushing patients with longstanding post-treatment proteinuria develop tubulo-interstitial injury and whether these drugs would also be indicated.

The high prevalence of proteinuria found in old Beagle dogs also warrants further investigation. We did not observe increased UPCs in the healthy control dogs included in our studies, but there are no large scale studies evaluating proteinuria in aged dogs of breeds other than Beagles.<sup>72</sup>

### **5. General conclusion**

Throughout this thesis we have gained significant knowledge on renal function in dogs with Cushing's syndrome. Furthermore, we have investigated the use of urinary markers in canine patients. First, sampling methods and sample storage conditions were evaluated, and assay validation was performed to ensure optimal measurement of urinary markers. Then uALB, uCRP, uRBP and uNAG were evaluated in healthy young and old dogs, and in dogs with CKD. This study showed that the urinary markers were increased in dogs with CKD compared to healthy dogs, and that there was no difference between young and aged healthy dogs. In the second part of this thesis we evaluated renal function in dogs with Cushing's syndrome in a cross-sectional study pre-treatment and a longitudinal study pre- and post-treatment with trilostane or transsphenoidal hypophysectomy. Patients with Cushing's syndrome had significantly higher UPC, glomerular (uALB/c and uIgG/c) and tubular urinary markers (uRBP/c and uNAG/c), which declined following treatment, indicating reversible combined HMW and LMW proteinuria in most dogs. However, almost 40% of dogs remained proteinuric at the end of the one year follow-up period. Pre-treatment GFR measured by plasma  $Cl_{\text{creat}}$  and  $Cl_{\text{endo}}$  was significantly higher in untreated dogs with Cushing's syndrome and declined post-treatment. None of the patients developed CKD during the study period, but a low  $Cl_{\text{creat}}$  was detected in about 30% of dogs at the end of the study. We did not detect significant differences for  $Cl_{\text{exo}}$ , indicating that dispositions of the two stereo-isomers may vary according to the clinical setting. Similar functional changes in GFR and proteinuria were detected in older Beagle dogs treated with exogenous HC. Renal lesions and proteinuria were present in many dogs before the start of HC administration. At the end of the HC treatment period, glomerular and tubulo-interstitial lesions had progressed in both the treatment and control group. Therefore, additional studies are needed to elucidate GC effects on renal morphology.



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## **SUMMARY**

## *Summary*



**Summary**

Cushing's syndrome is a common endocrine disorder in middle aged to older dogs. Proteinuria occurs in 44 to 75% of affected dogs and is a sign of kidney dysfunction. Moreover, there is evidence that protein overload in the ultrafiltrate may be toxic to the tubular cells and triggers pathways that cause tubulo-interstitial injury. Another frequent complication in up to 80% of human and canine Cushing patients is hypertension, which can also be detrimental to the kidney. Because of its similarity with the syndrome in humans and its high estimated incidence compared to the low incidence in people, canine Cushing's syndrome is an interesting animal model for its human counterpart. Surprisingly, very little scientific information exists on renal consequences of Cushing's syndrome in people as well as in dogs.

Routine markers such as serum creatinine (screat) and urea only detect renal disease when up to 75% of kidney function is already lost. Hence the need for markers that allow early detection of renal dysfunction. Glomerular filtration rate (GFR) is considered the best overall index of renal function. In human medicine, certain urinary markers allow early and site-specific indication of renal dysfunction.

Aims of this thesis were to evaluate renal function in dogs with Cushing's syndrome using routine renal parameters as well as GFR and urinary markers.

At the start of this research, only few studies had described measurement of urinary markers in veterinary medicine. Therefore, information about sampling methods and urine sample storage conditions was mandatory. In a first study (**Chapter III**), we evaluated effects of sampling method (voided urine versus cystocentesis) and storage temperature (-20° compared to -80°) on urinary concentrations of albumin (ALB), retinol-binding protein (RBP) and N-acetyl-β-D-glucosaminidase (NAG). Urinary marker concentrations were not influenced by sample type. However, caution may be warranted, because one dog had markedly elevated urinary (u) ALB, uRBP and uNAG concentrations in the voided sample. Samples for analysis of uALB and uRBP can be stored at -20°C for up to 4 months, whereas -80°C is preferred for storage up to 12 months. Urinary NAG enzyme activity is less stable at -20°C as well as -80°C, with already substantial losses after 4 months at -20°C and a more severe decline after 12 months at -80°C.

Because of the paucity of knowledge on the use of urinary markers in dogs, solid assay validation was required as well as information about normal values and concentrations in dogs

## **Summary**

with kidney disease. In the second study (**Chapter IV**), our objectives were to validate a canine ALB ELISA and a uNAG colorimetric test and to compare two glomerular markers, uALB and uCRP, and two tubular markers, uRBP and uNAG, between healthy and chronic kidney disease (CKD) dogs. A third aim was to detect a possible age-related difference in these markers between healthy young and older dogs. Assay characteristics of the ALB ELISA and NAG colorimetric test were satisfactory for both assays. Urinary glomerular and tubular markers were higher in dogs with CKD than in healthy controls and there was no difference in urinary marker concentrations between healthy young and older dogs. Although the number of dogs was too small to define actual reference ranges for the urinary markers, the study in Chapter IV provided essential information on assay validation, and urinary marker concentrations in healthy dogs and in dogs with CKD. Because Cushing's syndrome occurs in middle-aged to old dogs, the absence of an age-related difference in the urinary markers was an important finding.

In the second part of the thesis, we applied the urinary markers as well as GFR to evaluate renal function in dogs with Cushing's syndrome (**Chapter V**). We conducted a cross-sectional study in untreated dogs (§ 5.1) and a longitudinal follow-up study (§ 5.2) before and after treatment of Cushing's disease with trilostane or transsphenoidal hypophysectomy. Patients with Cushing's syndrome had significantly higher UPC, glomerular (uALB/c, uIgG/c) and tubular urinary markers (uRBP/c and uNAG/c), which decreased after treatment, indicating reversible combined HMW and LMW proteinuria in most dogs. However, proteinuria persisted in 43% of dogs treated with trilostane and in two dogs with recurrence of PDH after hypophysectomy, but not in any of the other surgically treated dogs. Post-treatment decrease of urinary markers was also less pronounced in the medically treated than in the surgically treated dogs, suggesting that the latter may be less prone to persistent proteinuria. Pretreatment GFR measured by plasma  $Cl_{\text{creat}}$  and  $Cl_{\text{endo}}$  was significantly higher in untreated dogs with Cushing's syndrome and declined post-treatment. None of the patients developed CKD during the study period, but a low  $Cl_{\text{creat}}$  was detected in four of 13 dogs at the end of the study. Significant changes in  $Cl_{\text{exo}}$  were not detected, indicating that the two iohexol stereo-isomers need to be considered as different markers.

Finally, in **Chapter VI** we investigated changes in GFR and urinary markers in old Beagle dogs before and after treatment with hydrocortisone, including renal biopsies. Similar to our findings in patients with Cushing's syndrome, GFR was higher after hydrocortisone

treatment in these Beagle dogs and proteinuria tended to increase. Renal lesions and proteinuria were present in many dogs before the start of hydrocortisone administration, which raises questions for interpretation of similar findings in dogs with glomerular diseases. Moreover, this important observation has to be kept in mind when using Beagle dogs as a model for human renal disease or toxicological studies. At the end of the hydrocortisone treatment period, glomerular and tubulo-interstitial lesions had progressed in both the treatment and control group. Therefore, additional studies are needed to elucidate glucocorticoid effects on renal morphology.

The main findings of the thesis are the following:

- Urine samples for measurement of uALB and uRBP can be stored at  $-20^{\circ}\text{C}$  for up to 4 months, whereas  $-80^{\circ}\text{C}$  is preferred for storage up to 12 months. For uNAG, storage at  $-80^{\circ}\text{C}$  and analysis within 4 months is advised.
- Effects of sampling method on urinary marker concentrations (voided compared to cystocentesis) seems to be minimal, after urine sample centrifugation and in absence of UTI.
- Intra- and inter-assay CV, limit of detection and linearity of a canine uALB ELISA and a uNAG colorimetric test were determined.
- uALB/c, uRBP/c and uNAG/c are significantly increased in dogs with CKD (IRIS stages II to IV) and their concentrations do not differ between healthy young and aged dogs.
- Pretreatment, urinary glomerular (uALB and uIgG) and tubular markers (uRBP and uNAG), UPC and GFR measured with plasma  $\text{Cl}_{\text{creat}}$  and  $\text{Cl}_{\text{endo}}$  are significantly increased in dogs with Cushing's syndrome.
- Post-treatment, there is a decline of GFR, without development of azotemia in any of the dogs during a 12 month follow-up. Persistent proteinuria was detected in 43% of dogs treated with trilostane and in none of the dogs treated with hypophysectomy and without recurrence.
- Apparently healthy aged Beagle dogs may have significant histopathological renal lesions and proteinuria.
- Chronic administration of hydrocortisone causes an increase in GFR in Beagle dogs.



## **SAMENVATTING**

## *Samenvatting*

**Samenvatting**

Cushing's syndroom is een vaak voorkomende endocriene aandoening bij honden van middelbare tot oude leeftijd. Proteïnurie komt voor bij 44 tot 75% van de gevallen en is een teken van een verstoorde nierfunctie. Bovendien zijn er studies die suggereren dat een overmaat aan proteïnen in het ultrafiltraat toxisch zou zijn voor de tubuluscellen en aanleiding kan geven tot een cascade reactie die tubulo-interstitiële schade veroorzaakt. Een andere frequente complicatie bij ongeveer 80% van de mensen en honden met het syndroom van Cushing is hypertensie, wat ook schadelijk kan zijn voor de nier. Omdat dit syndroom zeer gelijkaardig is bij honden en mensen, maar bij de hond een veel hogere geschatte incidentie heeft, is Cushing's syndroom bij de hond een interessant diermodel voor de humane tegenpool. Verrassend genoeg bestaat er zeer weinig informatie over de gevolgen van Cushing's syndroom voor de nier, zowel bij mensen als bij honden.

Routinemerkers zoals serum creatinine (screat) en ureum detecteren een daling in de nierfunctie pas als deze reeds met 75% verminderd is. Er is daarom nood aan merkers die vroegtijdige detectie van een gedaalde nierfunctie toelaten. De glomerulaire filtratiesnelheid (GFS) wordt beschouwd als de beste manier om de algemene nierfunctie te evalueren. In de humane geneeskunde zouden bepaalde urinaire merkers vroege detectie en lokalisatie van een verstoorde nierfunctie mogelijk maken.

Het doel van deze thesis was om de nierfunctie bij honden met Cushing's syndroom te evalueren aan de hand van routineniermerkers evenals GFS en bepaalde urinaire merkers.

Bij het begin van dit onderzoek waren er in de diergeneeskunde slechts een beperkt aantal studies die meting van urinaire merkers beschreven. Informatie ontbrak over het nemen van de urinestalen en de optimale condities om deze te bewaren. In een eerste studie (**Hoofdstuk III**) evalueerden we het effect van staalname (urine via mictio in vergelijking met cystocentese) en bewaartemperatuur (-20°C in vergelijking met -80°C) op de urinaire concentraties van albumine (ALB), retinol-bindingsproteïne (RBP) en N-acetyl-β-D-glucosaminidase (NAG). De concentraties van deze urinaire merkers werden niet beïnvloed door het type van staalname. Deze bevinding moet echter voorzichtig geïnterpreteerd worden, omdat er bij één hond duidelijk gestegen uALB, uRBP en uNAG concentraties werden teruggevonden in het mictio staal. Monsters voor analyse van urinair (u) ALB en uRBP kunnen tot 4 maanden bewaard worden bij -20°C, terwijl -80°C aangewezen is voor langere bewaring tot één jaar. Enzymactiviteit van uNAG is minder stabiel bij -20°C alsook bij -

## ***Samenvatting***

80°C, met al substantiële daling na 4 maanden bij -20°C en een nog sterkere afname na één jaar bij -80°C.

Door de beperkte kennis over het gebruik van urinaire merkers bij honden was een solide validatie van de testkits nodig, evenals meer informatie over normaalwaarden bij gezonde honden en concentraties bij honden met nierziekte. De doelstelling van een tweede studie (**Hoofdstuk IV**) was om een caniene ALB ELISA en uNAG colorimetrische test te valideren voor gebruik met urine. Verder werden twee glomerulaire merkers, uALB en uCRP, en twee tubulaire merkers, uRBP en uNAG, vergeleken tussen gezonde honden en honden met chronische nierziekte (CNZ). Ten derde werden deze urinaire merkers vergeleken bij gezonde jonge en oude honden om na te gaan of er een leeftijdsgerelateerd verschil was. Alle analytische karakteristieken van de ALB ELISA en uNAG colorimetrische test voldeden aan de vooropgestelde criteria. Urinaire glomerulaire en tubulaire merkers waren hoger bij honden met CNZ dan bij gezonde honden en er was geen verschil in urinaire merker concentraties tussen gezonde jonge en oude honden. Hoewel het aantal honden te klein was om effectief referentie intervallen te definiëren, zorgde de studie in Hoofdstuk IV voor essentiële informatie over validatie van de tests en concentraties van de urinaire merkers bij gezonde honden en honden met nierziekte. Omdat Cushing's syndroom voorkomt bij honden van middelbare tot oude leeftijd was het ontbreken van een leeftijdsgerelateerd verschil bij de urinaire merkers een belangrijke bevinding.

In het tweede gedeelte van de thesis werden de urinaire merkers alsook de GFS gebruikt om nierfunctie bij honden met Cushing's syndroom te evalueren (**Hoofdstuk V**). Er werd een studie bij onbehandelde honden uitgevoerd (§ 5.1) en een longitudinale opvolgingsstudie (§ 5.2) voor en na behandeling van de ziekte van Cushing met trilostane of transspenoidale hypofysectomie. Patiënten met Cushing's syndroom hadden significant hogere eiwit/creatinine ratio's (e/c), glomerulaire (uALB/c en uIgG/c) en tubulaire urinaire merkers (uRBP/c en uNAG/c), die afnamen na de behandeling, wat duidt op een reversibele proteïnurie bij de meeste honden. De proteïnurie bleef echter aanwezig bij 43% van de honden behandeld met trilostane en bij twee honden met een herval van de ziekte van Cushing na hypofysectomie. Geen enkel van de chirurgisch behandelde honden had persisterende proteïnurie. De urinaire merkers daalden minder sterk na behandeling bij de medisch behandelde honden, wat er eventueel op zou kunnen wijzen dat deze laatste vaker persisterende proteïnurie hebben dan honden na hypofysectomie. Deze hypothese moet echter verder onderzocht worden bij een grotere groep honden en met randomisatie. De GFS vóór



behandeling, gemeten aan de hand van  $Cl_{creat}$  en  $Cl_{endo}$  was significant hoger bij de onbehandelde honden met Cushing's syndroom en daalde na de behandeling. Geen van de patiënten ontwikkelde CNZ gedurende de studieperiode, maar een lage  $Cl_{creat}$  werd gevonden bij vier van de 13 honden op het einde van studie. Significante veranderingen van  $Cl_{exo}$  werden niet teruggevonden, wat er op wijst dat de twee stereo-isomeren van iohexol als twee verschillende merkers moeten beschouwd worden.

Tot slot werden in **Hoofdstuk V** GFS-veranderingen, urinaire merkers en nierbiopten bij oude Beagles onderzocht, voor en na behandeling met hydrocortisone. Gelijkaardig aan de resultaten bij patiënten met Cushing's syndroom, was de GFS hoger na behandeling met hydrocortisone en was er een stijgende trend in de proteïnurie. Nierletsels en proteïnurie waren aanwezig bij veel honden voor de start van de hydrocortisone toediening, wat vragen oproept over de interpretatie van dergelijke bevindingen bij honden met glomerulaire ziekten. Bovendien moet deze belangrijke observatie in acht genomen worden wanneer Beagles gebruikt worden als model voor humane nierziekten of toxicologische studies. Aan het einde van de hydrocortisone behandeling was er progressie in de glomerulaire en tubulo-interstitiële letsels zowel als bij de controle groep als bij de behandelde honden. Er zijn dus bijkomende studies nodig om de effecten van glucocorticoïden op de niermorfologie op te helderen.

Samenvattend kan men uit deze thesis het volgende besluiten:

- Urinemonsters voor meting van uALB en uRBP kunnen tot 4 maanden bewaard worden bij  $-20^{\circ}\text{C}$ , terwijl  $-80^{\circ}\text{C}$  aangewezen is voor bewaring tot één jaar. Voor uNAG wordt bewaring bij  $-80^{\circ}\text{C}$  met analyse binnen de 4 maanden aangeraden.
- Type van staalname (mictio in vergelijking met cystocentesis) lijkt minimale effecten te hebben op urinaire merker concentraties, na centrifugatie van de monsters en in afwezigheid van een urineweginfectie.
- Intra- en inter-assay variatie coëfficiënt, detectielimiet en lineariteit van een canine uALB ELISA en uNAG colorimetrische test werden bepaald.
- uALB/c, uRBP/c en uNAG/c zijn significant gestegen bij honden met CNZ (IRIS stages II tot IV) en hun concentraties verschillen niet tussen gezonde jonge en oude honden.
- Voor behandeling zijn urinaire glomerulaire (uALB en uIgG) en tubulaire merkers (uRBP en uNAG), e/c en GFS, gemeten met  $Cl_{creat}$  en  $Cl_{endo}$ , significant gestegen bij honden met Cushing's syndroom.

## *Samenvatting*

- Na behandeling is er een daling van de GFS zonder dat één van de patiënten azotemie ontwikkelde gedurende de studieperiode van één jaar. Persisterende proteïnurie werd teruggevonden bij 43% van de honden behandeld met trilostane, terwijl geen enkele van de patiënten behandeld met hypofysectomie, zonder herval, persisterende proteïnurie vertoonde.
- Schijnbaar gezonde oude Beagles kunnen duidelijke histopathologische nierletsels hebben en proteïnurie.
- Chronische toediening van hydrocortisone veroorzaakt een stijging van de GFS bij Beagles.

**DANKWOORD**

*Dankwoord*

Een citaat van de Engelse schrijver Samuel Johnson klinkt als volgt:

“What is written without effort is, in general, read without pleasure.”

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# **CURRICULUM VITAE**

## *Curriculum vitae*

Pascale Smets werd geboren op 3 april 1982 te Leuven.

Zij behaalde in 2000 het diploma algemeen secundair onderwijs Latijn-wetenschappen aan het Heilig Hartinstituut te Heverlee.

Nadien startte ze de studie Diergeneeskunde aan de Universiteit van Gent. In 2006 behaalde ze met grote onderscheiding het diploma van Dierenarts in de afstudeerrichting Gezelschapsdieren. Onmiddellijk daarna volgde zij een roterend Internship bij de vakgroep Geneeskunde en Klinische Biologie van de Kleine huisdieren aan de faculteit Diergeneeskunde.

Geboeid door de mogelijkheden die het wetenschappelijk onderzoek kan bieden voor de kliniek, startte zij een doctoraatsstudie naar nierfunctie bij honden met het syndroom van Cushing. Deze studie werd gefinancierd door het Fonds voor Wetenschappelijk Onderzoek Vlaanderen en het Bijzonder Onderzoeksfonds van de universiteit Gent. Tevens vervulde zij in 2011 het trainingsprogramma van de Doctoral School of Life Science and Medicine van de Universiteit Gent.

Pascale Smets is auteur of mede-auteur van 20 wetenschappelijke publicaties en abstracts. Zij nam actief deel aan meerdere internationale congressen.

## ***Curriculum vitae***

### **Publications in peer-reviewed international journals**

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## *Curriculum vitae*

### **Research communications/abstracts presented during international scientific meetings:**

Smets, P., Lefebvre, H.P., Meij, B.P., Meyer, E., van de Maele, I., Daminet, S., 2011. Long-term follow-up of renal function in dogs with Cushing's disease before and after treatment. Oral presentation at the 21<sup>st</sup> congress of the European College of Veterinary Internal Medicine (ECVIM). Proceedings of the 21<sup>st</sup> ECVIM congress, September 8<sup>th</sup>-10<sup>th</sup>, Seville, Spain, p. 195. *Awarded the ESVE MSD Animal Health prize for best oral presentation 2011.*

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