CANINE ADRENAL GLAND LESIONS: FROM ULTRASONOGRAPHY TO LABORATORY FINDINGS

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ABBREVIATIONS

ACL adrenocortical lesions

ACTH adrenocorticotropic hormone

ADL aldosterone

AGL adrenal gland lesions

ATS functional adrenocortical tumor

CI confidence interval

HAC hyperadrenocorticism

HDDST high-dose dexamethasone suppression test

HPLC high-pressure liquid chromatography

LC-MS liquid chromatography tandem mass spectrometry

LDDST low-dose dexamethasone suppression test

MN metanephrine

NAL nonadrenal lesions

NMN normetanephrine

PDH pituitary-dependent hyperadrenocorticism

PHEO pheochromocytoma

UCCR urinary cortisol:creatinine ratio

US ultrasonography

CONTENTS

Chapter 1: AIMS AND SCOPE OF THE THESIS

The first descriptions of the ultrasonographic appearance of normal canine adrenal glands were published in the mid-1990s (Barthez et al. 1995; Douglass et al. 1997). Prior to this time, the available technology did not support the routine identification of adrenal glands in healthy dogs (Kantrowitz et al. 1986; Kantrowitz et al. 1990). Since those first reports, the ability of an experienced sonographer to visualize the adrenal glands has continuously improved, and the identification, description, and measurement of both adrenal glands is now considered a standard part of an abdominal ultrasonographic routinely examination in dogs. Therefore, adrenal gland lesions are now routinely detected in dogs, and for veterinarians defining the importance of these lesions became challenging (Barthez et al. 1997; Myers et al. 1997; Melian et al. 2000). Advanced imaging modalities, such as CT, MRI, may provide useful additional information, but require anaesthesia, long lead time and additional costs (Rosenstein et al. 2000; Schultz et al. 2009). Focal adrenal gland lesions (nodules and masses) or nonspecific enlargement may reflect one of many diseases. A lesion may be a hyperplastic lesion (ie, a cortical or medullary nodular hyperplasia) or benign tumor (ie, a cortical adenoma or myelolipoma) or malignant tumor (ie, a cortical carcinoma, pheochromocytoma, or metastases). Rarely, it may be a granuloma, cyst, or adrenalitis with lymphoplasmacellular or purulent infiltrates (Barthez et al. 1997; Besso et al. 1997; Barrera et al. 2013). Incidentally discovered adrenal masses are becoming more common with the development of imaging techniques and constitute a significant clinical problem. In veterinary medicine, the prevalence of adrenal gland lesions in dogs examined via abdominal ultrasonography is described to be 4%, similar to that reported in human patient populations undergoing abdominal CT (Cook et al. 2014). Results of previous studies (Besso et al. 1997; Hoerauf et al. 1998) suggested that ultrasonographic findings have limited value for differentiation between benign and malignant, cortical and medullary, secreting and not-secreting lesions. For these reasons, a multidisciplinary approached has been proposed, with the interpretation of ultrasonographic images in conjunction

with clinical findings, biochemical and endocrine tests results (Besso et al. 1997). Algorithms for endocrine testing and imaging procedures are not currently available for investigating the underlying causes of adrenal lesions in dog and an accurate management requires an agreement among results of these diagnostic procedures. The evaluation of new biomarkers, as plasma-free metanephrines or serum inhibin, for the diagnosis of adrenal lesion origins started recently. These parameters in association with imaging procedures were able to distinguish dogs affected by pheochromocytoma from dogs with adrenal cortical lesions or nonadrenal lesions and healthy dogs (Salesov et al. 2015). Further research is needed to further define appropriate criteria for a diagnostic workup of dogs with adrenal gland lesions (AGL). The goals of this study were:

1. describe the prevalence of AGL in dogs undergoing routine diagnostic abdominal ultrasonography
2. assess the accuracy of ultrasound in the evaluation of adrenal glands size and differentiation between the different types of AGL
3. evaluate the accuracy of recent biochemical markers in the differentiation of AGL origins in dogs.

Chapter 2: General introduction

ANATOMY

Topographic Anatomy

The adrenal glands (*glandula suprarenalis*) are located near the cranio-medial border of each kidney (**Figure 1**). The right adrenal gland is retroperitoneal, near the hilus of the corresponding kidney. Its longer segment projects caudally, along the caudal vena cava, and the shorter segment projects caudo-laterally toward the cranial pole of the right kidney. Its firm connective tissue attachments bring it into close proximity with the caudal vena cava as its immediate medial boundary. Often, the capsule of the right adrenal gland is continuous with the tunica externa (adventitia) of the caudal vena cava. The right common trunk for the caudal phrenic and cranial abdominal arteries crosses its dorsal surface, and the mass of the right kidney covers this adrenal gland on its ventro-lateral surface. As a result of these organ relationships, this gland assumes a triangular or wedgeshape in transectional profile. Its ventral surface is bisected by the common venous trunk accompanying the above artery, and the cranial two thirds of this adrenal gland is covered by the caudal extension of the right lateral hepatic lobe of the liver. Both glands lie in a generous bed of retroperitoneal fat. The left adrenal gland is also retroperitoneally positioned near the cranio-medial border of the left kidney. This adrenal gland is firmly bound in the loose collagenous connective tissue of the fascia. Thus it is more structurally related by position to the abdominal aorta than to the left kidney. Medially, it is bounded by the abdominal aorta at a position just caudal to the origin of the cranial mesenteric artery and adjacent to the origin of the common trunk for the caudal and cranial phrenico-abdominal arteries. This latter vessel courses over its dorsal surface at the midpoint of the gland. The caudal border of the left adrenal gland is limited by the renal artery and vein. Its ventral surface is bisected by the common venous trunk accompanying

the above artery, and is covered to varying degrees by the spleen. Laterally, its boundary is formed by the kidney (Hullinger et al. 1978)

Macroscopic Anatomy

Both adrenal glands are composed of three portions: cranial pole, middle portion (hilus) and caudal pole, as showed in **Figure 2**. The left adrenal gland has a cranial pole that is somewhat flattened dorsoventrally and oval in outline, and its caudal pole is cylindrical. The right adrenal gland has an acute angular bend with its cortex projecting cranially. Baker (1936) reported that adult males of mixed breeds have adrenal glandular tissue weighing approximately 1.14 g. The female in diestrus has glandular tissue weighing approximately 1.24 g. In general, the left and right glands differ in weight, but this difference has not been found to be statistically significant. The adrenal cortex usually completely invests the adrenal medulla. The medulla comes closest to the outer surface at the hilus of each gland. Subtle and easily overlooked, the hilus is located near the middle portion of the medial surface and serves as the exit point for the adrenal vein or veins.

Microscopic Anatomy and Cytophysiology

The adrenal gland is composed of two structurally and functionally different tissues: an outer cortex and an inner medulla (**Figure 3**). The outer most zone of the adrenal cortex is the zona arcuata or zona glomerulosa (ZG). It is composed of cells arranged in arches and nestled in a stromal template provided by the inner surface of the capsule. The next cortical zone, the zona fasciculata (ZF), is the thickest. Its narrow outer surface is called zona intermedia corticalis. This small region comprises less than 5% of the total cortex and functions as a blastemic region for replacement cells of the adult cortex (Nussdorfer 1986, Hullinger 1978). The cells of the outer one-third of this zone contain more lipid and are somewhat larger than those of the inner two-thirds. The innermost cortical layer is applied to all surfaces of the undulating contour of the medulla. The parenchyma of this inner zone is disposed in a relatively random and loose network and is termed the zona reticularis (ZR).

The medulla, the central core of the gland, is separated from the cortex by a delicate network of reticular and loose collagenous connecting tissues, the septum corticomedullae. The adrenal cortex is a major steroid-producing organ. The ZG of the canine adrenal cortex secretes mineralocorticoids (primary aldosterone, ALD) and is deficient in 17α-hydroxylase activity (CYP17), which renders it incapable of synthesizing cortisol or androgens. In contrast, only cells in the ZG contain the enzymes necessary to synthesize ALD. The ZF and ZR function as a unit, secreting glucocorticoids (mainly cortisol) and small amounts of sex steroids (estrogens, progestins, and androgens). The adrenal medulla secretes catecholamines (adrenaline and noradrenaline) (O'Brien 2001).

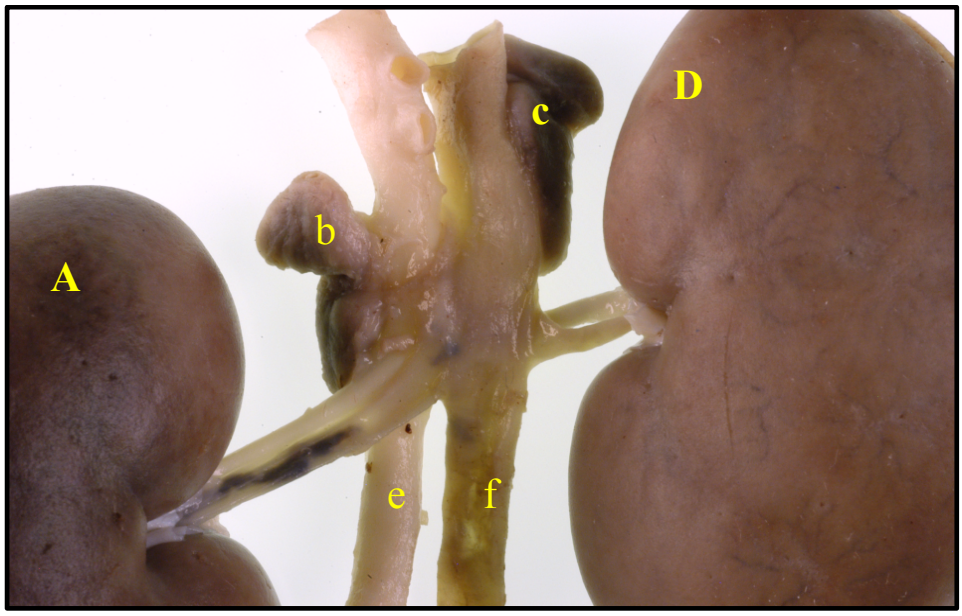

Figure 1: The adrenal glands, ventral aspect. **A**: left kidney; **b**: left adrenal gland; **c**: right adrenal gland; **D**: right kidney; **e**: aorta; **f**: caudal vena cava

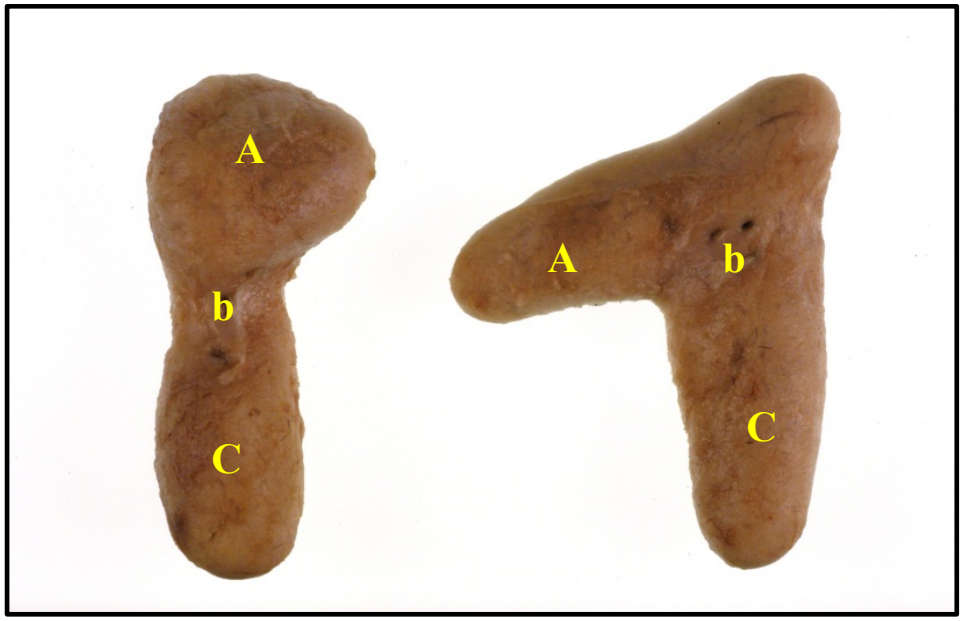

Figure 2: Macroscopic Features of left and right adrenal glands (**A**: cranial pole; **b**: middle portion (hilus) ; **C**: caudal pole)

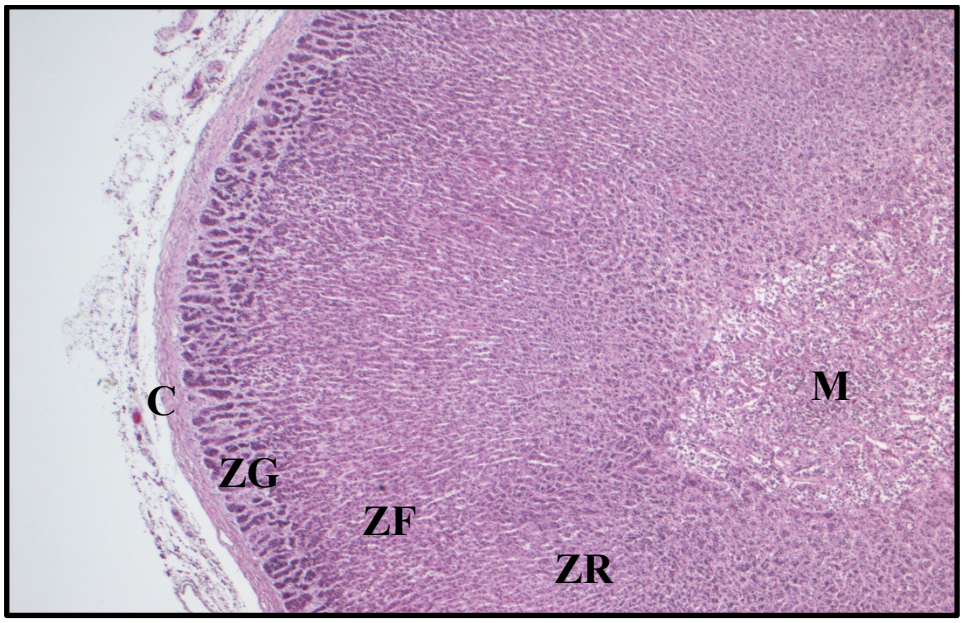

Figure 3: Mesoscopic Features of left adrenal gland of a normal and mature dog. Seven years old Cocker Spaniel, trasverse section. Hematoxylin eosin staining method **(C**: capsule; **ZG**: zone arcuata o glomerulosa; **ZF**: zone fasciculata; **ZR**: zone reticularis; **M**: medullary)

PHYSIOLOGY

Glucocorticoids

The secretion of glucocorticoids is under the control of the hypothalamic-pituitary-adrenocortical axis (HPA), which represents a complex set of direct influences and feedback interactions between the hypothalamus, the pituitary gland, and the adrenal glands. The main pituitary hormone regulating adrenal steroid secretion is the adrenocorticotropic hormone (ACTH). In the neuroendocrine control of ACTH secretion the following mechanisms can be distinguished: episodic secretion, response to stress, feedback inhibition of cortisol, and immunological factors (Galac et al. 2010). Like the other hormones of the pituitary, ACTH is secreted in a pulsatile way and consequently also the cortisol. Central nervous system events regulate both the number and magnitude of ACTH pulses, ranging in the dog from six to twelve per 24 hours period (Kooistra et al. 1997a). There seems to be no circadian rhythm in cortisol secretion in dogs (Kemppainen and Sartin 1984, Kooistra et al. 1997a, Koyama et al. 2003). Both ACTH and cortisol are secreted within minutes following the onset of stress (Benson et al. 2000). Indeed, using urinary cortisol as an integrated measure of cortisol production, stress such as exposure to veterinary procedures is reflected in elevated urinary corticoid:creatinine ratios (UCCR) (Van Vonderen et al. 1998). Feedback inhibition is another major regulator of ACTH and thus cortisol secretion. The inhibitory action of glucocorticoids is exerted at multiple target sites, of which two have been identified: neurons in the hypothalamus that produce corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP), and corticotropic cells in the pituitary gland. On the other hand, challenges to the immune system by infection invariably activate the HPA. This response is mediated by proinflammatory cytokines, a group of polypeptides released from colonies of activated immune cells. Among the several cytokines, particularly interleukin (IL-6) activates the HPA (Dunn 2000). The regulatory actions of the cytokines are exerted predominantly at the level of the hypothalamus, where CRH is the major mediator of the hypothalamic response. Cytokine mediated activation of

the HPA is also subject to feedback regulation by glucocorticoids, which not only impair the hypothalamic response to cytokine activation but also block cytokine production and inhibit macrophages. Thus there is bidirectional communication between the neuroendocrine system and the immune system (John and Buckingham 2003).

The glucocorticoids produced by the adrenal glands after the pituitary stimulation, cannot be stored in steroidogenic cells but are secreted immediately after biosynthesis. Cortisol, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone, and aldosterone are derived almost exclusively from adrenal secretion, whereas most other steroids are derived from a combination of adrenal and gonadal sources (O`Brien 2001). In the adrenocortical cell, glucocorticoid biosynthesis is initiated by binding of ACTH to its receptor (ACTH-R) that is mainly expressed in the adrenal cortex. All steroid hormones are derived from cholesterol. The adrenocortical cells can synthesize cholesterol de novo from acetate, mobilize intracellular cholesterol ester pools, or import lipoprotein cholesterol from plasma. About 80% of the cholesterol is usually provided by circulating plasma lipoproteins (Gwynne and Hess 1980). Under normal conditions, cholesterol de novo synthesis from acetyl coenzyme A represents about 20% of steroidogenic capacity. The conversion of cholesterol to pregnenolone is the first and rate limiting step in the cortisol steroidogenic pathway (**Figure 4**). It occurs in the mitochondria and involves the action of the cholesterol-side-chain cleavage enzyme (CYP11A). The second step is conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase-type 2 (HSD3B), while the following step is catalyzed by CYP17, which has both 17α-hydroxylase activity and 17,20-lyase activity. This dual function allows the enzyme to direct steroid precursors along several different pathways: 17α-hydroxylated substrates with the side chain are glucocorticoid precursors (17-OH progesterone and 17-OH pregnenolone), whereas both 17α-hydroxylase and 17,20-lyase direct substrate toward androgen and estrogen synthesis (dehydroepiandrosterone and androstenedione). Both progesterone and its 17α-hydroxylated derivate undergo 21-hydroxylation by a single 21-hydroxylase (CYP21) in the smooth

endoplasmic reticulum. The last step in cortisol biosynthesis is 11β-hydroxylation of 11-deoxycortisol, catalyzed by 11β- hydroxylase-type 1 (CYP11B1). For this step, 11-deoxycortisol is transferred from the smooth endoplasmic reticulum back to the mitochondria (Galac 2010).

Mineralocorticoids

Aldosterone (ALD) is the most potent mineralocorticoid and the three main mediators of its release are the renin-angiotensin system (RAS), potassium, and ACTH (Corry and Tuck 2001). In particularly, the most sensitive mediator of renin release is renal perfusion pressure, changes in which are sensed by myotransducers in the wall of the afferent arteriole. The resulting signals are transmitted to the juxtaglomerular apparatus to modify the level of renin release and, therefore, ALD secretion (Bader and Ganten 2000). Among the factors inhibiting renin release, angiotensin II and potassium play the most important roles. Angiotensin II directly suppresses renin release through a short feedback loop, independent of changes in blood pressure, ALD secretion, or renal blood flow. Potassium acts directly on the ZG cells by activating the conversion of cholesterol to pregnenolone (Corry and Tuck 2001). It suppresses renal renin release by directly inhibiting the release of renin-containing granules. Furthermore, potassium is a very potent stimulus for ALD secretion and small increases in plasma potassium concentration produce an immediate and significant increase in plasma ALD concentration (Williams 2005). Adrenocorticotropin hormone (ACTH) is a potent, acute ALD secretagogue, but in both humans and experimental animals, the effect of ACTH infusion is short-lived unless it is pulsatile (Seely et al. 1989). During continuous ACTH stimulation, glomerulosa cell function appears to convert to fasciculata cell function and thus long-term ACTH stimulation paradoxically decreases ALD secretion. The secretion of ALD is restricted to the ZG. The biosynthetic pathway of ALD is initially similar to that of cortisol except that 17-hydroxylation does not occur, because of the lack of CYP17. In ALD synthesis progesterone is metabolized in the endoplasmic reticulum to 11-deoxycorticosterone. This is transferred to the mitochondria, where the zone specific 11β-hydroxysteroid dehydrogenase-type 2

(CYP11B2 or ALD synthase) converts it to corticosterone and progressively oxidizes it to ALD (O`Brien 2001) (**Figure 4**).

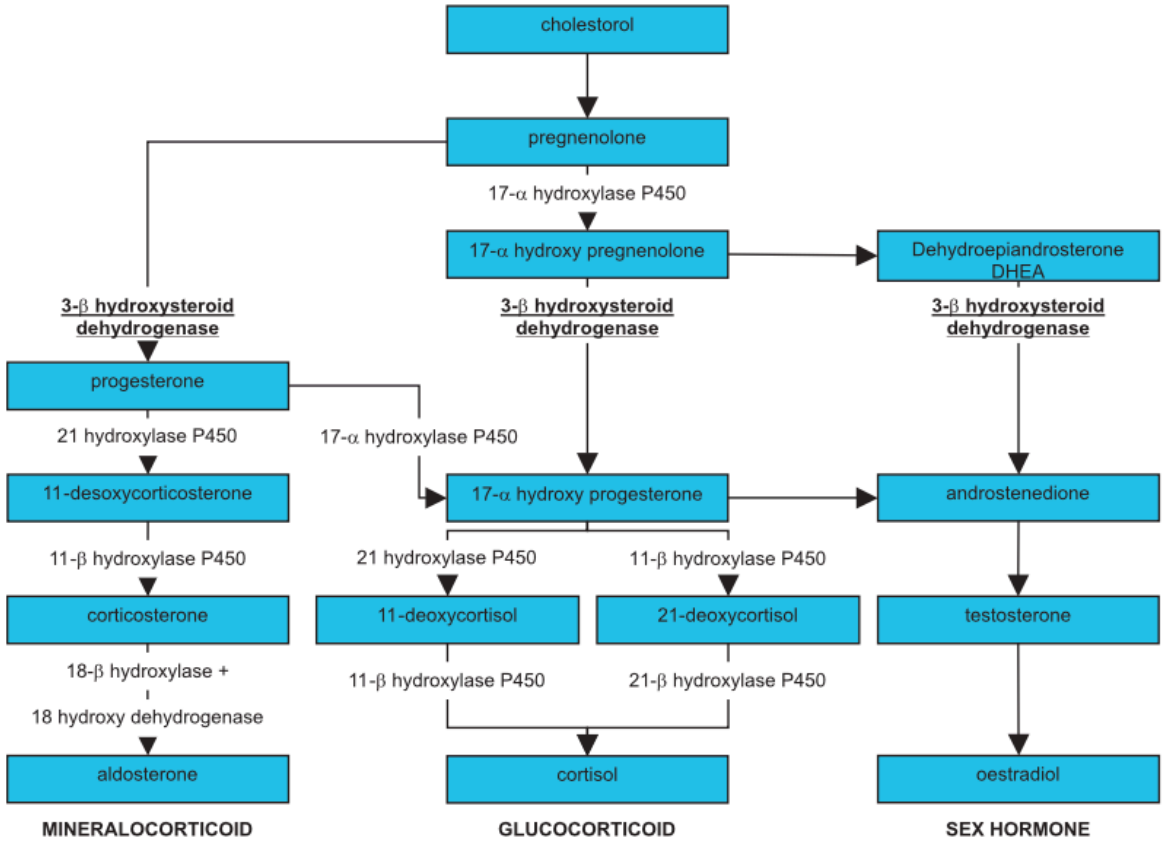

Figura 4: Schematic representation of adrenal steroid biosynthesis.

Cathecolamines

The catecholamines, epinephrine (adrenalin), norepinephrine (noradrenalin), and dopamine, are synthesized in the adrenal medulla, the sympathetic nervous system, and the brain. As they influence virtually all tissues and are involved with other hormonal and neuronal systems in the regulation of a wide variety of physiological processes (Eisenhofer et al. 2004).

In the adrenal medulla, chromaffin cells are specialized for the synthesis, storage, and secretion of catecholamines. Synthesis of norepinephrine in these cells follows the basic steps:

1. tyrosine's hydroxylation to dopa

2. dopa's decarboxylation to dopamine

3. transport of dopamine into the vesicles

4. dopamine's hydroxylation to norepinephrine

The adrenal medulla, transforms about 80% of the norepinephrine into epinephrine, through the following reaction:

5. norepinephrine's methylation to epinephrine

Chromaffin cells are innervated by preganglionic sympathetic neurons in the splanchnic nerves, and, because of their unique blood supply, are exposed to unusually high concentrations of glucocorticoids in the venous drainage from the adrenal cortex. Splanchnic nerve stimulation appears to be the most important determinant of adrenomedullary function. Splanchnic nerve stimulation leads to an increase in the activity of several of the catecholamine biosynthetic enzymes, and to an increase in the rate of catecholamine biosynthesis. Glucocorticoids cause the induction of the enzyme noradrenaline N-methyltransferase, and so are particularly important for the synthesis of epinephrine. Catecholamines are stored, together with ATP, Ca2+, and protein, in secretory vesicles known as chromaffin granules. Splanchnic nerve stimulation is the physiological stimulus for catecholamine secretion. Stimulation of the splanchnic nerves results in the release of acetilcoline from nerve endings in the adrenal medulla. Acetilcoline causes an increase in the permeability of the chromaffin cells to Ca2+, and thereby leads to the entry of Ca2+ into the cells. Ca2+ then causes the secretion of catecholamines and of other chromaffin granule constituents from the chromaffin cells by exocytosis. Once the catecholamines enter the circulation, they can exist in the plasma in free form or in conjugation with other substances (Eisenhofer et al. 2004).

Ordinarily, the norepinephrine secreted directly into a tissue remains active for only a few seconds, demonstrating that its reuptake and diffusion away from the tissue are rapid. However, the norepinephrine and epinephrine secreted into the blood by the adrenal medullae remain active until they diffuse into some tissue, where they can be destroyed by catechol-O methyl transferase; this occurs mainly in the liver. Therefore, when secreted into the blood, both norepinephrine and

epinephrine remain very active for 10 to 30 seconds; but their activity declines to extinction over 1 to several minutes (Guyton et al. 2006).

Primary adrenal gland tumors are relatively infrequent in dogs, reported in approximately 0.17% to 0.76% of pet dogs (representing 1%-2% of all canine tumors). Among these, adenoma and adenocarcinoma are the most common, followed by pheochromocytoma, myelolipoma, aldosteronoma and deoxycorticosterone-secreting and sex hormone–secreting adrenal tumors (Massari et al. 2011). A retrospective study that included all dogs that underwent complete necropsy evaluation during a 20-year period at the University of California–Davis reported that 195 (41%) of 472 neoplastic canine adrenal lesions were adrenocortical tumors, 151 (32%) were pheochromocytomas, and 126 (27%) were metastatic lesions (Labelle et al. 2005).

Most adrenocortical tumors in dogs are functional. Increased glucocorticoid levels are responsible for the constellation of clinical signs and biochemical abnormalities associated with Cushing's syndrome. Hyperadrenocorticism is the most commonly diagnosed endocrinopathy in dogs. Therefore, the evaluation of clinical and historical signs of cortical hormonal overexpression should be the first step of the diagnostic workup. On the other hand, there is no widely used test for the diagnosis of pheochromocytoma in dogs, and affected patients show no consistent abnormalities on CBC, biochemical profiles, or urinalysis that makes the diagnosis more challenging.

Hyperadrenocorticism

Hyperadrenocorticism or hypercortisolism or Cushing`s syndrome is one of the most common endocrinopaties in dogs. There are three major causes of hyperadrenocorticism in dogs: by oversecretion of ACTH by the pituitary gland, cortisol-secreting adrenocortical neoplasia or iatrogenic administration of glucocorticoids (Feldman et al. 2004). In about 80-85% of dogs, hyperadrenocorticism results from hypersecretion of adrenocorticotropic hormone (ACTH) by a pituitary corticotroph adenoma and is called ACTH- or pituitary-dependent hyperadrenocorticism (PDH). In the remaining cases, canine hyperadrenocorticism is ACTH-independent and arises from

excessive secretion of glucocorticoids by a benign or malignant adrenocortical tumor (AT_S). In humans, hyperadrenocorticism may also a result from ectopic secretion of either ACTH or CRH, but in dogs is not commonly recognized (Newel-Price et al. 1998).

ACTH-dependent hyperadrenocorticism

Pituitary-dependent hyperadrenocorticism

Pituitary-dependent hyperadrenocorticism (PDH) is one of the most common endocrine disorders in dogs. Some epidemiological studies have estimated the incidence to be 1 to 2 cases/1,000 dogs/year (Willeberg and Priester 1982), while in humans it is a rare disorder with an estimated incidence of 1.2–2.4 new cases/million/year (Lindholm et al. 2001). The excessive secretion of ACTH, resulting in a bilateral adrenocortical hyperplasia, can be due to a pituitary microadenoma, macroadenoma, corticotroph hyperplasia, or rarely adenocarcinoma. Most pituitary tumors in dogs with pituitary-dependent hyperadrenocorticism are microadenomas, defined as a tumor less than 10 mm of diameter. Only about 10 to 15% of dogs have larger corticotropic adenomas (macroadenomas) at the time of diagnosis (Feldman et al. 2004).

Hyperadrenocorticism due to ectopic ACTH secretion

Ectopic ACTH syndrome accounts for about 15% of cases of ACTH-dependent hyperadrenocorticism in humans. The majority of the ectopic tumors that secrete ACTH originate from cells of the diffuse neuroendocrine system and are highly malignant. They include small lung carcinomas, thymic and bronchial carcinoids, pancreatic islet cell tumors, medullary carcinomas of the thyroid, and pheochromocytomas (Newell- Price et al. 1998). The rapidly developing physical changes are usually associated with extremely high plasma concentrations of ACTH and cortisol and often severe hypokalaemia. In humans circulating levels of cortisol are usually higher in the ectopic ACTH syndrome than in other forms of hyperadrenocorticism. This form of hyperadrenocorticism is often difficult to distinguish from PDH because tumors causing the ectopic

ACTH syndrome are frequently small and difficult to visualize (Newell-Price et al. 1998). Indeed, in 10-20% of human patients with ectopic ACTH syndrome the source of ACTH hypersecretion remains occult, in spite of extensive investigation and prolonged follow-up. In such cases, medical treatment is applied to control hypercortisolemia and the diagnostic imaging is repeated after some time (Grossman et al. 2006). In one study, ectopic ACTH secretion was described in a dog with hyperadrenocorticism and abdominal neuroendocrine tumors (Galac et al. 2005).

ACTH-independent hyperadrenocorticism

Cortisol-secreting adrenocortical tumors

ACTH-independent hyperadrenocorticism in dogs is usually caused by cortisol-secreting adrenocortical tumors (ATs). Adrenocortical tumors are responsible for approximately 15 to 20% of dogs with Cushing's syndrome. Most ATs are unilateral lesions and the left or right adrenal glands may be affected equally. Bilateral tumors occur in about 10% of cases (Ford et al. 1993, Hoerauf et al. 1999). Functional adrenocortical tumor secrete cortisol independent of pituitary ACTH control. Through the negative-feedback effects of glucocorticoids on the pituitary gland, the excess cortisol secreted by the adrenal tumor chronically suppress endogenous ACTH secretion, resulting in atrophy of the contralateral non pathological adrenal gland (Feldman et al. 2004).

Histologically, ATs are classified as adenomas or carcinomas, although the distinction can be difficult. Adrenocortical adenomas are usually small, well-circumscribed tumors that do not metastasize and are not locally invasive. In contrast, adrenocortical carcinomas are usually large, locally invasive, hemorrhagic, and necrotic, with distant metastasis reported to occur in only 5% to 14% of cases (Anderson et al. 2001, Kyles et al. 2003, Schwartz et al. 2008). Tumor calcification also occurs in over 50% of dogs with adrenal carcinoma (Reusch et al. 1991). Carcinomas, especially of the right adrenal gland, frequently invade the phrenicoabdominal vein and caudal vena cava and metastasize to the liver, lung, and kidney. While expansion of tumor tissue into blood

vessels is generally considered to be a hallmark of malignancy, ATs may be an exception to this rule (Van Sluijs et al. 1995), because a microscopic examination of a seemingly benign tumor may reveal its expansion into blood vessels (Labelle et al. 2004). In humans a scoring system which takes into account not only the results of histopathological examination but also the clinical picture and follow-up has been introduced to improve the reliability of the differential diagnosis of AT (Weiss et al. 1989; Volante et al. 2009). The most frequent modulation in human ATs is overexpression of the IGF-II gene, observed in 85% of carcinomas (Gicquel et al. 2001, Fottner et al. 2004), and one of the major characteristics of carcinomas is expansive growth, which is negatively correlated with steroid secretion (Mendonca et al. 1995). Furthermore, carcinomas have been found to be unable to carry steroidogenesis to completion, resulting in plasma patterns of steroid precursors typical of enzymatic blockage of cortisol synthesis with resulting hypersecretion of adrenal sex hormones (Syme et al. 2001, Hill et al. 2005). Mixed cortisol- and aldosterone-producing ATs have also been reported in dogs (Behrend et al. 2005, Machida et al. 2008). Simultaneous occurrence of an AT and a pheochromocytoma (Von Dehn et al. 1995) or/and pituitary adenoma (Greco et al. 1999, Thuròczy et al. 1998) has also been described in dogs.

Iatrogenic Hyperadrenocorticism

Iatrogenic hyperadrenocorticism results in clinical signs and physical examination findings similar to others forms of natural hyperadrenocorticism. Excessive or prolonged administration of corticosteroids causes iatrogenic hyperadrenocorticism. Because of the negative-feedback effects of glucocorticoids on the pituitary gland, endogenous ACTH production is suppressed, resulting in atrophy of both adrenal cortices (Peterson et al. 2007)

History, clinical signs and physical examination

Pituitary-dependent hyperadrenocorticism is usually a disease of middle-aged to older dogs, with a median age of approximately 10 to 11 years (Feldman et al. 2004). Dogs with ACTH-independent

hyperadrenocorticism tend to be slightly older, with a median age of 11 to 12 years (Reusch et al. 1991). There is no gender predilection. It occurs in all dog breeds, with a predilection for small breeds, such as dachshunds, poodles and miniature terriers. Adrenocortical tumors occur more frequently in larger breeds with about 50% of dogs weighing greater than 20 kg (Reusch et al. 1991). Many of the clinical signs can be related to the biochemical effects of glucocorticoids (gluconeogenesis and lipogenesis at the expense of protein). The most common clinical signs associated with hyperadrenocorticism in dogs are polydipsia, polyuria, polyphagia, lethargy, abdominal enlargement or potbelly, panting, obesity, muscle weakness, and recurrent urinary tract infections. Dermatologic manifestations of hyperadrenocorticism commonly include truncal hair thinning or alopecia. The polyuria is known to be due to impaired osmoregulation of vasopressin release and interference by the glucocorticoid excess with the action of vasopressin in the kidney (Galac et al. 2010). In one study, 86% of dogs with uncontrolled hyperadrenocorticism were hypertensive. The exact mechanism of the development of systemic hypertension in dogs with hyperadrenocorticism is unknown, but it seems to be related to the mineralicorticoid-like effect of cortisol which, under normal physiologic conditions, is insignificant, but, in patients with cortisol excess, becomes clinically significant (Ortega et al. 1996).

More rare clinical signs have been described in dogs affected by PDH and ATs. Clinical changes associated with the pituitary origin of the disease are only observed when the pituitary tumor becomes large enough to cause neurological symptoms. These are often vague, consisting of lethargy, inappetence and mental dullness (Wood et al. 2007). Clinical findings linked exclusively to an AT may be related to the adrenal mass and/ or caused by metastases or nonspecific features of malignancy such as weight loss and anorexia. A palpable abdominal mass, obstruction of the caudal vena cava by tumor thrombus (Jaffe et al. 1999), or hemoperitoneum secondary to rupture of the tumor (Whittemoore et al. 2001).

On physical examination, the most commonly noted abnormalities include abdominal enlargement, hepatomegaly, panting, truncal obesity, bilaterally symmetric alopecia, comedomes, pyoderma, and seborrhea. Hyperpigmentation, thin skin, bruising, and calcinosis cutis are less commonly recorded (Peterson et al. 2007).

Routine Laboratory Tests

In dogs, increased serum alkaline phosphatase (ALP) activity is a frequent laboratory alteration with hyperadrenocorticism, which is observed in 85-90% of dogs (Feldman et al. 2004). High serum alanine transferase activity (ALT), hypercholesterolemia, hyperglycemia, and low blood urea nitrogen are also common findings. The hemogram may reveal a mild erythrocytosis as well as a classic "stress leukogram" (ie, eosinopenia, lymphopenia, and mature leukocytosis). The urine-specific gravity is usually less than 1.015. Dogs with hyperadrenocorticism can usually concentrate their urine if water is deprived, but their concentrating ability is frequently reduced. Rarely, in some dogs with a pituitary macroadenoma, compression of the posterior lobe of the pituitary and suprasellar extension into the hypothalamus may cause disruption to antidiuretic hormone production and release resulting in signs of central diabetes insipidus. Finally, many dogs with hyperadrenocorticism have evidence of urinary tract infection with proteinuria resulting from glomerulosclerosis (Peterson et al. 2007).

Hormonal Tests

The diagnosis of hyperadrenocorticism should be made from clinical signs, physical examination, routine laboratory tests, and diagnostic imaging findings, but the diagnosis must be confirmed by hormonal tests (Feldman et al. 2004). The hormonal tests should demonstrate the increased production of cortisol and decreased sensitivity to glucocorticoid feedback (Miller and Crapo 1994). The most commonly used hormonal tests are: the corticotropin (ACTH) stimulation test, low-dose dexamethasone suppression test, and the urinary cortisol:creatinine ratio. None of these tests are

perfect, and all of them can give false-negative and false-positive test results. The definitive diagnosis of hyperadrenocorticism should never be made purely on the basis of results of one or more of these screening tests, but the interpretation of the diagnostic imaging findings should be made in conjunction with clinical aspects and results of biochemical and endocrine tests.

The measurement of **basal serum or plasma cortisol concentration** has little diagnostic value because the pulsatile secretion of ACTH results in a fluctuate blood cortisol concentration throughout the day, resulting in high degree of overlap of values in normal dogs, dogs with nonadrenal illness and dogs with hyperadrenocorticism. The sensitivity of basal cortisol determinations in the diagnosis of hyperadrenocorticism is only about 50%, whereas the specificity can be extremely low in dogs with severe nonadrenal illness (Peterson et al. 2007). Cortisol values are only useful after dynamic stimulation with ACTH or suppression after dexamethasone.

The **low-dose dexamethasone suppression test** (LDDST) is considered to be the test of choice for the diagnosis of hyperadrenocorticism in dogs (Behrend et al. 2002). Dexamethasone is used for the low-dose dexamethasone suppression test because it is a potent glucocorticoid, which does not cross-react with the standard cortisol assays, allowing one to use serum (plasma) or urine measurements. To perform this test, serum or plasma cortisol concentrations are determined before, 4 and 8 hours after the administration of the dexamethasone preparation. Either a solution of dexamethasone in polyethylene glycol (0.015 mcg/kg, IV or IM) or dexamethasone sodium phosphate (0.01 mcg/kg, IV) can be administered for the test with equivalent results. In normal dogs, intravenous administration of 0.01 mg dexamethasone per kg body weight suppresses plasma cortisol concentration to 40 nmol/L or less at 8 hours after dexamethasone administration (Greco et al. 1993, Kemppainen and Peterson 1993). In dogs with hyperadrenocorticism there is little or no suppression at 8 hours (Feldman et al. 2004). The LDDST is a reliable screening test with a reported sensitivity of 85% - 100%, and a specificity of 73% (Rijnberk et al. 1988, Kaplan et al.

1995). The disadvantage of the LDDST is that it is rather time consuming and invasive, requiring blood collection at least three times.

Another commonly used hormonal test for hyperadrenocorticism is the **urinary cortisol-to-creatinine ratio** (UCCR). Because urine accumulates in the bladder for several hours before collection, its corticoid concentration is an integrated measure of corticoid production over this interval, smoothing out the effects of short-term fluctuations in plasma cortisol concentration. The urinary corticoids (largely cortisol) are related to the creatinine concentration in the urine, resulting in the UCCR. To avoid the influence of stress, urine for measurement of the UCCR should be collected at home, with an interval of at least one day after the visit to the veterinary clinic. The owner collects a sample of the first morning urine on two consecutive days and the UCCRs in these two samples are averaged. In dogs the predictive value of a positive test result is 0.88 and that of a negative test result is 0.98 (Rijnberk et al. 1988). This test requires little time, is not invasive (no blood collection), and has a high diagnostic sensitivity. In addition, the test procedure has the advantage of combining a test for basal adrenocortical function and a dynamic test for differential diagnosis.

Cortisol urinary evaluation and an oral LDDST can be done in combination. The owner collects urine at 8.00 h (at home) for measurement of the basal UCCR and then administers 0.01 mg dexamethasone per kg body weight orally. The dog is walked at 12.00 h and 14.00 h to empty its bladder and then urine is collected at 16.00 h to measure the effect of the low dose of dexamethasone on the UCCR (Feldman 2005).

In dogs in which results of the UCCR and/or the LDDST have been inconclusive or negative but in which there is still suspicion of hyperadrenocorticism, the **ACTH stimulation test** can be used. In principle it is a test of adrenocortical reserve capacity, used to diagnose primary and secondary adrenocortical insufficiency, and thus it can be used to diagnose iatrogenic hyperadrenocorticism,

which via feedback suppression results in secondary adrenocortical insufficiency. However, the ACTH stimulation test was used more often in the past as a primary test for hyperadrenocorticism.

The basis for this test is that dogs with pituitary-dependent hyperadrenocorticism or cortisol-secreting adrenal tumors, because of their increased adrenocortical mass, have the capacity to secrete excessive amounts of cortisol that could be identified with the ACTH test.

The method for ACTH stimulation testing in dogs is to determine serum cortisol concentrations before and 1 hour after the intravenous or intramuscular injection of synthetic ACTH. In normal dogs, administration of a supraphysiological dose of ACTH produces arise in serum cortisol to values usually greater than 10 mcg/dL (>300 nmol/L) and lessen than 20 mcg/dL (<600 nmol/L). In contrast, dogs with hyperadrenocorticism tend to have an exaggerated response to ACTH administration, with post-ACTH serum cortisol concentrations rising to greater than 20 mcg/dL (>600 nmol/L). The ACTH stimulation test identifies over half of dogs with cortisol-secreting adrenocortical tumors and about 85% of dogs with pituitary-dependent hyperadrenocorticism (Peterson et al. 1982). Based on these results, the sensitivity of the ACTH stimulation test in diagnosing pituitary-dependent hyperadrenocorticism can be calculated to be 85%, whereas the sensitivity is only 60% in dogs with secerning adrenal-cortical tumors. The main advantages of the ACTH stimulation test are its simplicity and short duration. Furthermore is the only screening test than can identify dogs with iatrogenic hyperadrenocorticism. However, its diagnostic accuracy for hyperadrenocorticism is less than that of the UCCR and the LDDST.

Some dogs have classic clinical signs of hyperadrenocorticism and typical hematological and biochemical findings but have a normal cortisol response to both ACTH stimulation and low-dose dexamethasone suppression. These cases have been termed atypical hyperadrenocorticism. It has been suggested that cases of atypical hyperadrenocorticism may have a derangement of the steroid production pathway and that some of the precursors of cortisol, such as 17-hydroxyprogesterone, may be abnormally increased. Plasma 17-hydroxyprogesterone concentrations show an exaggerated

response to ACTH stimulation in both typical and atypical hyperadrenocorticism (Benitah et al. 2005; Hill et al. 2005).

Once the diagnosis of hyperadrenocorticism has been confirmed, it is necessary to distinguish between PDH and hyperadrenocorticism due to ATs. Despite decreased sensitivity to suppression by glucocorticoids, which is a hallmark of hyperadrenocorticism, in most dogs with PDH due to an adenoma in the anterior lobe, ACTH secretion can still be suppressed by a high-dose of dexamethasone (Feldman et al. 2004). In contrast, PDH of pars intermedia origin is resistant to suppression by dexamethasone. In PDH, the resistance to glucocorticoid feedback is significantly correlated with the size of the pituitary gland (Kooistra et al. 1997b, Bosje et al. 2002). The impaired sensitivity to glucocorticoid feedback in PDH due to an anterior lobe tumor can be demonstrated by performing an intravenous high-dose dexamethasone suppression test (HDDST). The high-dose dexamethasone suppression test is performed in a manner similar to that of the low-dose suppression test with respect to sample collection timing. The recommended protocols for the high-dose dexamethasone suppression test is to administrate 0.1 to 1.0 mg/kg, IV or IM of dexamethasone. A decrease of more than 50% from the baseline value confirms PDH (Stolp et al. 1983). When the suppression is less than 50%, the hyperadrenocorticism may still be pituitary-dependent (due to either a pars intermedia neoplasia or a resistant anterior lobe neoplasia) or be due to ATs. Further differentiation requires measurements of plasma ACTH concentration. In animals with PDH, plasma ACTH concentration is not completely suppressed despite high plasma cortisol concentrations (Gould et al. 2001). Endogenous ACTH concentrations are normal to high in dogs with pituitary-dependent hyperadrenocorticism (eg, >40 pg/mL or >8.8 pmol/L), whereas ACTH concentrations are usually low or undetectable (eg, >20 pg/mL or >4.4 pmol/L) in dogs with adrenal tumors or with iatrogenic hyperadrenocorticism. Unfortunately, about 20% of dogs with hyperadrenocorticism will have random plasma ACTH concentrations in the “gray zone” (ie, too

low for pituitary dependent disease but too high to be classified as adrenal dependent disease) (Peterson et al. 2007).

Diagnostic imaging

Abdominal radiography, abdominal ultrasonography, computed tomography and magnetic resonance imaging (of either the abdominal or the brain) can be used to diagnose and differentiate between pituitary-dependent hyperadrenocorticism and adrenal-dependent hyperadrenocorticism. In animals with adrenal disease, imaging is typically performed to confirm a tentative diagnosis based on clinical and laboratory findings

Conventional Radiography

Radiography has limited usefulness in the diagnosis because visualization of the adrenal and pituitary glands is hampered by inadequate resolution and superimposition of overlying tissues. The right and left adrenal glands are small retroperitoneal structures located at the level of the last thoracic and second lumbar vertebrae, respectively (Hullinger et al. 2010). Adrenal glands, however, cannot be seen with routine radiography unless enlarged or calcified (Pennick et al. 1988, Voorhout et al. 1988, Voorhout et al. 1990). An enlarged left adrenal gland may appear as a soft tissue mass or be inferred if the left kidney is displaced caudally. A mass effect created by an enlargement of the right adrenal gland is usually more difficult to identify because of its close proximity to the liver. Radiographic evaluation may be assisted using compression paddle techniques or linear tomography to relieve superimposed bowel, or angiography and intravenous urography to improve contrast of adjacent tissues. Prior to the development of CT, the pituitary gland could be evaluated indirectly using cavernous sinus venography and cerebral angiography. Pituitary tumors were suspected if displacement or deficient filling of nearby vessels was noted in the region of the hypophyseal fossa (Lee et al. 1972). These angiographic procedures have since been supplanted by CT and MRI and are not recommended because they are invasive and poorly

sensitive and specific. Despite its limitations, radiography provides useful information about the secondary effects of adrenal dysfunction as well as potentially identifying metastatic disease. Thoracic and abdominal radiographs are therefore recommended as part of the routine screening (Tidwell et al. 1997).

Abdominal ultrasonography

Ultrasonography is the most practical method of imaging the adrenal glands, because it is not time-consuming, reliable, noninvasive and does not require general anesthesia (Peterson et al. 2007). However, it is highly dependent on the quality of instrumentation and requires a high level of operator skill and diligence. The procedure usually entails real-time scanning in a parasagittal, dorsal, or oblique and transverse plane of the body to depict the long and transverse axes of the glands, respectively. The highest frequency transducer that allows adequate penetration should be used. A 5-MHz transducer is usually required for larger dogs, although compression of the ventral abdominal wall with the transducer may allow the use of a 7.5-MHz probe in compliant patients. Most animals are scanned in dorsal recumbency, after the hair is clipped and the skin is moistened with alcohol and acoustic gel. The left adrenal gland is identified by first locating the cranial pole of the left kidney in the parasagittal long-axis plane, then tilted the transducer medially until the aorta is visualizated. The left adrenal gland lies ventrolateral to the aorta and appears as a small peanut shell-shaped structure that is usually hypoechoic to surrounding retroperitoneal fat. Occasionally, an outer hypoechoic cortex can be distinguished from a hyperechoic medulla (**Figure 5**).

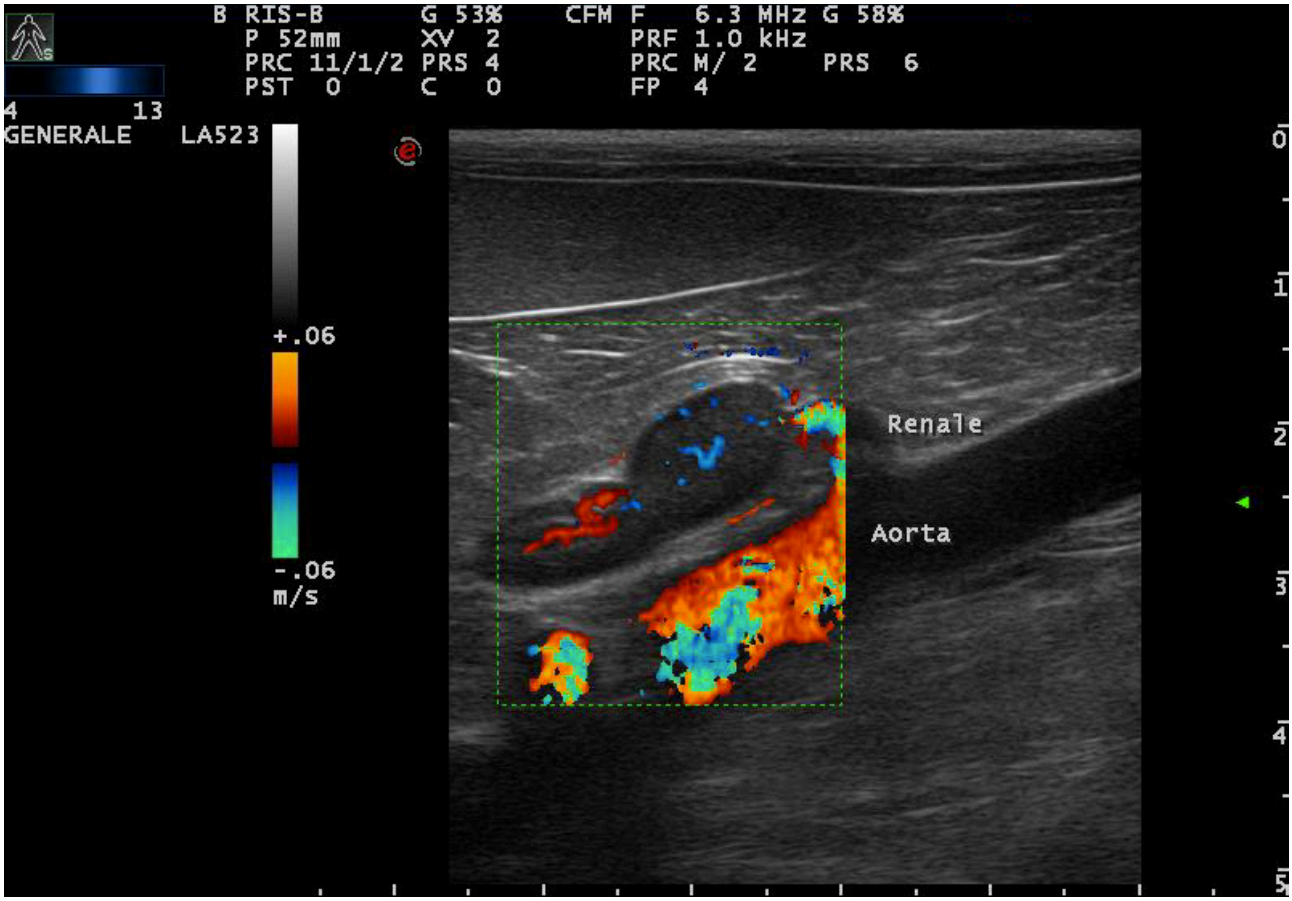

Figure 5: Left adrenal gland appears as a small peanut shell-shaped structure, ventrolaterally to the aorta.

The right adrenal gland may be identified with the patient in dorsal recumbency but tilted to the left, by fanning medially after locating the cranial pole or hilus of the right kidney in the parasagittal long-axis plane. Once the caudal vena cava is identified just caudal to the porta hepatis, the probe should be directed slightly laterally (**Figure 6**).

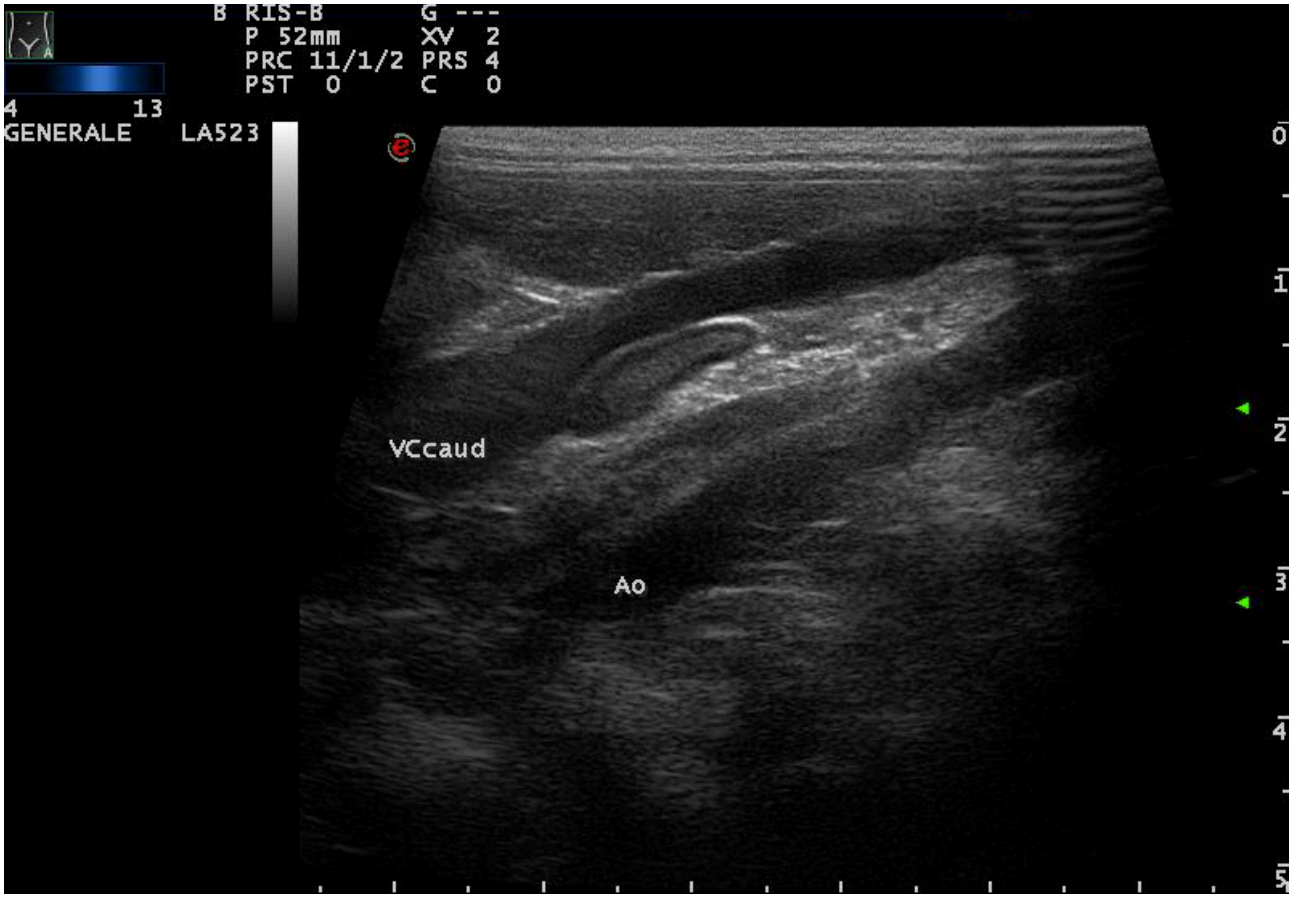

Figure 6: Right adrenal gland appears comma-shaped between the caudal vena cava and the aorta.

Depending on the approach, the gland may not be seen in the same image as the vessels and viewing the entire gland may require angling the transducer obliquely. As with the left adrenal gland, the right appears hypoechoic to surrounding fat or may have a layered appearance, but appears comma-shaped instead of peanut shell-shaped. Using a dorsal-plane right intercostal approach with the patient in left lateral recumbency, the right adrenal gland may appear as a triangular or comma-shaped structure on the near-field side of the eve. In the transverse plane, both adrenal glands appear oval and are located medial to the short axes of the kidneys (Tidwell et al. 1997). The left adrenal is located between the origin of the cranial mesenteric and left renal artery and the right adrenal just cranial to the right renal vein; both glands are enveloped by the phrenicoabdominal arteries and veins. However, these smaller vessels may not be readily seen without the aid of color-flow Doppler techniques. If bowel gas interferes with the acoustic window,

the animal should be repositioned or scanned from a different plane. Even then, one or both of the adrenal glands may not be visualized in normal patients. This is especially true for the right gland of larger dogs (Grooters et al. 1994).

Variable ultrasonographic measurements of normal and abnormal canine adrenal glands have been reported (Douglass et al. 1997, Grooters et al.1995, Grooters et al. 1996, Barthez et al. 1995, Barberet et al. 2010, Mogicato et al. 2011, Choi et al. 2012, de Chalus et al. 2013, Soulsby et al. 2014). Only one study evaluated the accuracy of ultrasonographic on the evaluation of adrenal gland measurements compared with their actual aspect. Moderate correlation was found between ultrasonographic and gross measurements of thickness (dorsoventral measure) for both left and right glands, but no correlation was found for width (mediolateral measure) or length (Grooters et al. 1995).

To minimize readers' misunderstanding, authors have decided to define "length" as the maximum craniocaudal dimension measured in the sagittal plane, "thickness" as the maximum dorsoventral dimension measured in the sagittal or transverse plane, and "width" as the maximum mediolateral dimension measured in the transverse plane (Soulsby et al. 2015) (**Figure 7-8**).

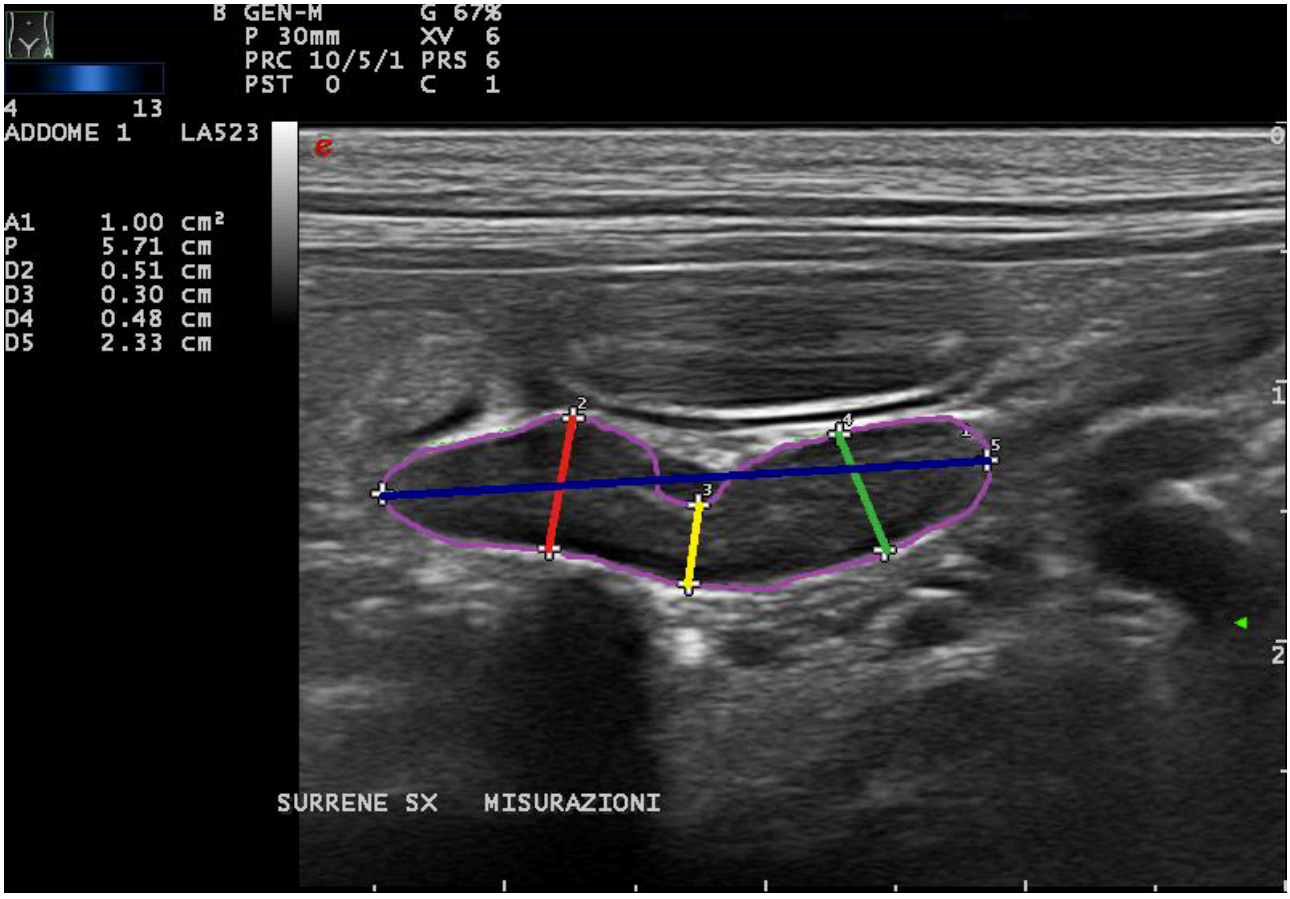

Figure 7: Ultrasonographic measurements of left adrenal gland in the longitudinal plan (A1: surface area; P: perimeter; D2: thickness of the cranial pole, D3: thickness of ileum, D4: thickness of caudal pole, D5: length)

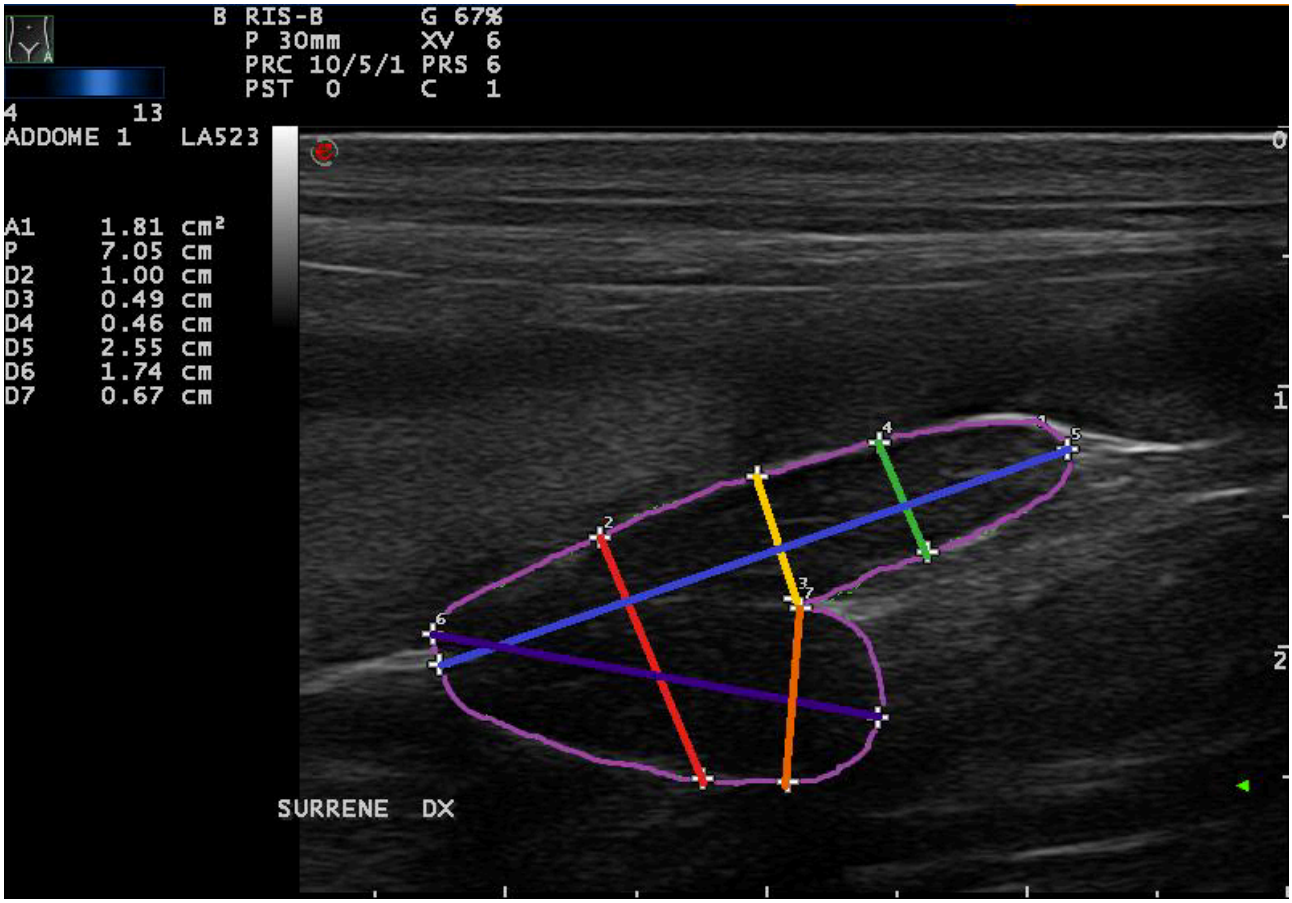

Figure 8: Ultrasonographic measurements of right adrenal gland in the longitudinal plan (A1: surface area; P: perimeter; D2: thickness of the cranial pole, D3: thickness of ileum, D4: thickness of caudal pole, D5: total length of the gland, D6: lenght of cranial pole, D7: width of cranial pole)

Some authors reported similar ranges of measurements in clinically healthy dogs without distintion between left and right adrenal glands (Barthez et al. 1998, Horauf et al. 1999 e Douglass et al. 1997):

- Thickness (longitudinal plan) ranging between 1,8 - 12,4 mm.
- Width (transversal plan), ranging between 3,6 - 8,1 mm.
- Lenght ranging between 10 - 50,2 mm.

Furthermore adrenal glands have been reported to have a thickness of 2–5 mm in normal young adult medium-sized dogs and 4–7 mm in middle aged to older dogs (Grooters et al. 1995, 1996). Grooters et al. (1995) have been suggested for normal adrenal glands:

- thickness of 3-6,5 mm for the left gland and of 1,8-6,7 mm for the right gland;
- width of 4-4,5 mm for the left gland and of 4 mm for the right;
- lenght of 23-25 mm for the left gland and of 22-25,8 mm for the right.

Mogicato et al. (2011) have been reported the following measurements:

- Thickness of 2,3-7,7 mm for the left gland and of 3-10,5 mm for the right gland;
- Lenght of 10,3-33,7 mm for the left gland and of 9,4-30,7 mm for the right gland;

Barberet et al. (2010), in study conducted on six healthy Beagles, have been reported:

- Thickness of 4,9-5,7 mm for the left gland and of 4,8-5,9 mm for the right gland;
- Lenght of 20,8-25 mm for the left gland and of 22,8-25,3 mm for the right gland;

A recent study (Choi et al. 2011) which analysed a population composed by small dogs (<10 kg) clinically healthy and affected by pituitary-dependent hyperadrenocorticism (PDH) described a median thickness of 4,32 +\- 1 mm for the left gland and of 4,21 +\- 1,1 mm for the right gland;

De Chalus et al. (2012) compared adrenal glands size of Yorkshire Terriers and Labrador Retrievers describing the following measurements:

For Yorkshire Terriers:

- Thickness (longitudinal plan) at the level of the cranial pole of 3,94 mm and at the level of the caudale pole of 4,14 mm.
- Width (transversal plan) at the level of the cranial pole of 6,44 mm and at the level of the caudale pole of 5,16 mm.
- Lenght of 14,89 mm

For Labrador Retrievers:

- Thickness (longitudinal plan) at the level of the cranial pole of 5,67 mm and at the level of the caudale pole of 6,29 mm.
- Width (transversal plan) at the level of the cranial pole of 11,1 mm and at the level of the caudale pole of 8,89 mm

- Lenght of 27,22 mm

Findings supported that adrenal glands size correlates with body weight in normal dogs. The length of the adrenal glands was associated with body weight, but not its thickness or width (Barthez et al. 1995, Douglass et al. 1997). On the other hand, thickness was described to be correlated with body weight by others studies (Grooters et al. 1995, 1998, Choi et al. 2011, de Chalus et al. 2012).

Soulsby et al. (2014) have been examined adrenal glands measurements in fuction of body weight in healthy clinically dogs divided in three categories (< 10 Kg, 10-30 Kg, >30 Kg) and references intervals have been provided.

Although diagnosis of adrenal gland dysfunction depends primarily on endocrinologic testing, ultrasonography has a potential role as a means of supporting a diagnosis of adrenal gland dysfunction and in distinguishing adrenal-dependent from pituitary-dependent hyperadrenocorticism (Kaplan et al. 1995; Behrend et al. 2013).

Ultrasonographic evaluation for adrenal endocrinopathy was mainly based on measurement of adrenal glands. For the diagnosis of pituitary-dependent hyperadrenocorticism, a threshold of 7.4 mm for adrenal width offers a reasonable combination of sensitivity and specificity (Barthez et al. 1995). The median (range) sensitivity was 77% (73–97%) and specificity was 94% (80–98%). More recently studies, based on canine weight categories, described a threshold for the diagnosis pituitary- dependent hyperadrenocorticism of 6.0 mm for left adrenal maximum diameter or of 5.4 mm for the thickness of caudal pole for dog < 10 kg of body weight; of 6.8 mm for the thickness of caudal pole for dog ranging 10 - 30 kg of body weight; and 8.0 mm for the thickness of caudal pole for dog > 30 kg of body weight (Choi et al. 2011; Soulsby et al. 2014). For the diagnosis of ATs, based on the results of Cook et al. (2014), malignancy should be strongly suspected in masses > 20.0 mm. This finding is consistent with a previous report that size of an adrenal mass is predictive of malignancy in dog. Although firm conclusions regarding the reliability of size as a predictor of biological behavior were limited by the small data set, it seems appropriate to recommend adrenal

glandectomy if an incidental adrenal lesion is > 20.0 mm (Besso et al. 1997; Cook et al. 2014). However functional adrenocortical tumors with dorsoventral diameter < 20.0 mm have been described relatively often (Gould et al. 2001; Besso et al. 1997; Hoerauf et al. 1999). One study provided a threshold of 5.0 mm of the maximum thickness to distinguish PDH from ATs in presence of equivocal adrenal asimmetry with the 95% confidence intervals for estimated sensitivity and specificity for ATs diagnosis were 82–100 and 82–99%, respectively (Benchekroun et al. 2010).

Other parameters in ultrasonographic diagnostic evaluation of adrenal glands, including variation in shape, echogenicity, laterality and presence of vascular invasion should be examinated.

Bilateral normalsized or large adrenal glands with normal shape and echogenicity are considered strong evidence of adrenocortical hyperplasia secondary to PDH (Gould et al. 2001; Grooters et al. 1996). Likewise, a large solitary and abnormally shaped adrenal mass invading adjacent structures or associated with distant metastases is highly indicative of ATs (Besso et al. 1997). Both focal adrenal gland lesions (nodules and masses) or nonspecific enlargement may reflect the presence of adrenal-dependent hyperadrenocorticism. A lesion may be a benign tumor (cortical adenoma) or a malignant tumor (cortical carcinoma). Mineralization and bilaterality was described to be present in both benign and malignant lesions. Heterogeneous parenchyma and focal areas of increased echogenicity previously have been described in adrenal glands of various shapes (normal, nodule, mass), harboring nodular adrenocortical benign lesions (Grooters et al. 1996; Besso et al. 1997).

Based on limited results, no definitive differentiation between benign and malignant lesions was using ultrasonographic criteria alone (Besso et al. 1997). Although evidence of vascular extension or the presence of a thrombus indicates malignancy and is often associated with cortical carcinomas, limited information is gained regarding biological behavior from ultrasonography alone (Barthez et al. 1997; Besso et al. 1997). Additional noninvasive diagnostic procedures, such as contrast-enhanced ultrasonography, could aid in the differentiation of adrenal gland adenomas from

other lesions in human patients (Friedrich-Rust et al. 2008). Although data regarding this imaging modality are limited in dogs, recent studies suggested that the technique is technically feasible and provides preliminary information regarding accuracy to discriminate malignant versus benign adrenal lesions with a sensitivity of 100.0% and a specificity of 80.0%. Results of these studies described that this method could be useful for differentiating between the different types of adrenal tumors in dogs (Pey et al. 2011, 2013; Bargellini et al. 2013, 2016).

However, apart from these typical ultrasonographic images, other images, such as equivocal adrenal asymmetry, can be more difficult to analyze for various reasons. First, adrenal asymmetry associated with an adrenal mass and contralateral atrophy is expected in unilateral ATs; however, to date, there is no established cut-off for defining adrenal atrophy in dogs (Benchekroun et al. 2010). Furthermore cases of hyperadrenocorticism caused by unilateral ATs have even been described with opposite gland thickness within reference values (Hoerauf et al. 1999). Additionally, the appearance of ATs themselves can sometimes differ from the enlarged, rounded, or irregularly rounded shapes with small and homogeneous functional nodules described (Besso et al. 1997; Hoerauf et al. 1999). Second, adrenocortical nodular hyperplasia involving one or both adrenal glands has been described in dogs with PDH, generating adrenal asymmetry to a variable extent, changes in adrenal shape and, in some cases, heterogeneous echogenicity of the adrenal gland. In such situations, the images can be confused with those observed in cases of ATs (Benchekroun et al. 2010). Ultrasonography also permits evaluation of other organs such as the liver, kidney, and spleen for secondary effects and/or for the presence of local or distant metastasis. It also facilitates percutaneous guided biopsy.

Computed tomography (CT)

In humans CT plays an important role in characterizing adrenal neoplasms (Blake et al. 2010; Johnson et al. 2009). The majority of adrenal adenomas and myelolipomas in humans contain a significant amount of intracellular fat, hence these benign tumors usually have lower x-ray

attenuation than malignant adrenal neoplasms (Blake et al. 2010) and the combination of low density values (<10 HU) in nonenhanced CT images and early contrast wash-in/washout through the mass seen in benign tumors, enables correct classification of benign versus malignant adrenal lesions in up to 96% of affected humans (Caoili et al. 2002). CT also enables detection of tumor- or blood clot-thrombus in vessels adjacent to the adrenal glands, which occurs as a result of invasion by malignant neoplasms. CT has also been found to be a useful method for imaging the adrenal glands in dogs. The CT appearance of adrenal glands has been described in healthy dogs, (Voorhout 1990a; Bertolini et al. 2006), dogs with pituitary-dependent hyperadrenocorticism (Bertolini et al. 2008; Wood et al. 2007; Van Der Vlugt-Meijer et al. 2002) and dogs with primary adrenal neoplasia (Morandi et al. 2007; Rosenstein et al. 2000; Schultz et al. 2009; Voorhout et al. 1990b). As in humans, CT is an accurate method for detection of vascular invasion by malignant adrenal neoplasms, with 92% sensitivity and 100% specificity in one study (Schultz et al. 2009). CT (Voorhout et al. 1990a) can reveal adrenal tumor expansion into blood vessels and possible metastasis in the other organs. If metastases are revealed, CT- guided needle aspiration biopsy can be performed. CT scan of the thorax should be made to determine whether there are metastases in the lungs. The histologic composition of adrenal neoplasms is somewhat heterogeneous in both humans and dogs with variable amounts of hemorrhage, necrosis and/or mineralization occurring in benign and malignant neoplasms (Johnson et al. 2009; Kawashima et al.1998). As a result, different tumor types may appear similar in CT (Morandi et al. 2007; Schultz et al. 2009); however, previous studies of the CT appearance of canine adrenal tumors have not investigated in detail their imaging-pathologic correlations. The canine pituitary gland can be visualized by computed tomography (CT). In dynamic contrastenhanced CT, the absence of the pituitary flush indicates atrophy of the neurohypophysis and the adenohypophysis. Displacement of the pituitary flush in the early phase of the dynamic CT can be used to identify and localize microadenomas originating from the AL or PI in dogs (Van der Vlught-Meijer et al. 2002, 2003).

Pituitary imaging using computed tomography is important to determine the pituitary dimensions and to confirm the presence of an adenoma (Meij et al., 1997a, Auriemma et al., 2009 and Kooistra and Galac, 2012). Pituitary imaging using contrast-enhanced CT is also a prerequisite for hypophysectomy since it enables the determination of the bony surgical landmarks in relation to the pituitary gland (Meij et al., 1997a and Meij et al., 1998). Pituitary imaging with CT allows the distinction between enlarged and non-enlarged pituitary glands (Kooistra et al., 1997). In dogs with pituitary-dependent hypercortisolism and non-enlarged pituitary glands, an isolated microadenoma was rarely directly visible on contrast-enhanced conventional CT (van der Vlugt-Meijer et al., 2003). In healthy dogs, dynamic contrast-enhanced CT allows the visualization of the neurohypophysis due to its early arterial enhancement, called the neurohypophyseal "flush" (van der Vlugt-Meijer et al., 2004). In dogs with pituitary-dependent hypercortisolism, the pituitary flush may be absent, displaced, or distorted, and this is considered indirect proof of a pituitary adenoma affecting the neurohypophyseal integrity (van der Vlugt-Meijer et al., 2003).

Nuclear magnetic resonance imaging (MRI)

Adrenal studies are readily performed with magnetic resonance imaging. Cases where ultrasonography has been unable to give a clear diagnosis are good candidates for MRI due to the superior resolution and contrast. Functional adrenocortical tumor can invade the vena cava and a definite vascular invasion can be difficult to ascertain from other imaging modalities. The ability of MRI to clearly image the structure in all three major planes allows for clear definition of invasion. These studies can aid in deciding whether lesions are infiltrative which may influence surgical excisability. Studies in the humans have found that with techniques to ascertain lipid content, the fat containing adrenal masses are generally benign with a high degree of specificity (Bagley et al. 2009).

Pituitary smaller tumors are more readily seen with MR imaging especially with dynamic contrast studies. Macroadenomas may enlarge dorsally and invade or compress the diencephalon.

Macroadenomas are usually hyperintense on T2-weighted sequences, and isointense on T1-weighted sequences. Most macroadenomas are enhanced following contrast administration. As the normal pituitary is naturally enhanced following contrast administration, some adenomas may actually not take up as much contrast as the normal surrounding tissues. Microadenomas, commonly associated with hyperadrenocorticism, maybe more difficult to appreciate for similar reasons, that is, the tumor is lost within the normally enhanced pituitary tumor (Gavin and Holmes 2009).

Treatment

The goal of treatment of hyperadrenocorticism is to eliminate the clinical signs related to glucocorticoid excess. Depending on the cause, this may be achieved by adrenalectomy, transsphenoidal hypophysectomy, or medical treatment with o,p'-DDD (mitotane) or trilostane or radiation theraphy.

The choice of therapy for hypercortisolism depends on the localisation of the problem (ATS versus PDH), the availability of more sophisticated methods (hypophysectomy and radiation therapy), motivation and budget of the owner and experience of the veterinarian.

Surgical treatment

The treatment of choice for AT is adrenalectomy, because the successful removal of the affected adrenal gland eliminates the tumor and thus the clinical signs related to glucocorticoid excess, without the need for lifelong medication. Because of the atrophy of the cortex of the nonpathological contralateral adrenal gland, due to the longstanding glucocorticoid excess, glucocorticoid substitution is needed temporarily (Galac et al. 2010). Adrenalectomy can be performed in dogs via a ventral midline celiotomy, with a paracostal extension of the incision when needed, or via a paracostal approach (Van Sluijs et al. 1995, Anderson et al. 2001, Kyles et al. 2003) or even via laparoscopic access (Mayhew et al. 2014). Open adrenalectomy is associated with a relative perioperative mortality rate in dogs ranging from 13.5% to 30% (Van Sluijs et al. 1995,

Anderson et al. 2001, Kyles et al. 2003, Massari et al. 2011, Schwartz et al. 2008), but patients surviving to discharge achieve good long-term outcome with a median survival time >10 months (Anderson et al. 2001, Kyles et al. 2003, Massari et al. 2011). Some studies have specifically addressed the predictors of outcome in dogs undergoing adrenalectomy: Anderson et al. (2001) reported that age, histologic type, and tumor size at diagnosis were not associated with survival time. Kyles et al. (2003) described that the presence of abdominal metastases identified at surgery did not influence outcome, and adrenalectomy with caval thrombectomy did not increase the mortality rate. Schwartz et al. (2008) found that concurrent nephrectomy was associated with a poor prognosis; thrombocytopenia, high BUN concentration and aspartate transaminase activity, and hypokalemia were associated with poorer outcome. More recently Massari et al. reported that survival time was significantly shorter for dogs with adenocarcinoma, tumor major axis length ≥ 5 cm, metastasis, and vein thrombosis (Anderson et al. 2001, Kyles et al. 2003, Massari et al. 2011, Schwartz et al. 2008). The relatively high risk of postoperative complications includes renal failure, pneumonia, pancreatitis, and pulmonary thromboembolism, but it also reflects the level of difficulty of the surgical procedure. In dogs with hyperadrenocorticism the adrenals are usually poorly accessible due to central obesity and hepatomegaly. Furthermore, ATs are friable, lie in close proximity to the vena cava, and tend to invade blood vessels. In conclusion, the skill of the surgeon can affect the outcome of the adrenalectomy. In humans, laparoscopic adrenalectomy has been reported to have lower perioperative morbidity and mortality than open transabdominal surgery (Lee et al. 2008). Only small numbers of laparoscopic adrenalectomies have been described in the veterinary medicine (Jiménez Pelàez et al. 2008, Smith et al. 2012, Mayhew et al. 2014), but from the results of these studies the surgical time and the post-operative hospitalization time seemed to be shorter compared to the open procedure (Mayhew et al. 2014).

The surgical treatment of PDH is the transphenoidal hypophysectomy, which is the only treatment that can eliminate the causative pituitary adenoma, but in the dog this requires complete removal of

the pituitary gland. As for adrenalectomy, in hands of a skilled neurosurgeon transphenoidal hypophysectomy is an effective treatment for canine PDH (Meij et al. 1998, 2002). Following hypophysectomy, hormone replacement therapy consists of lifelong administration of cortisone acetate and thyroxine and temporary administration of desmopressin, a synthetic vasopressin analogue. The major complications are postoperative mortality, hypernatremia due to acute AVP deficiency, prolonged central diabetes insipidus, keratoconjunctivitis sicca, and residual or recurrent hyperadrenocorticism (Meij et al. 1998). A study of the 10-year follow-up results in 150 dogs with PDH confirmed that it is effective treatment for PDH in dogs with nonenlarged pituitaries, especially in the long term, with remission for up to seven years (Hanson et al. 2005). However, the survival and disease-free periods decrease and the incidence of central diabetes insipidus increases with increasing pituitary size. Hence, transsphenoidal hypophysectomy can be expected to have the best outcome as the primary treatment in dogs with nonenlarged or only moderately enlarged pituitaries. Therefore, transsphenoidal hypophysectomy is expected to have the best outcome when there is an early diagnosis of PDH (Hanson et al. 2007).

Medical treatment

There are two treatments commonly used in the management of pituitary-dependent Cushing's disease in dogs: mitotane (o,p'-DDD) and trilostane.

Mitotane has historically been the drug most commonly used and the first management of canine pituitary-dependent hyperadrenocorticism with mitotane was first described over 20 years ago (Schechter et al. 1973). Mitotane is an adrenocorticolytic agent with a direct cytotoxic effect on the adrenal cortex, resulting in progressive necrosis and atrophy (Peterson 2001). Two protocols have been described for PDH in dogs: selective (Kintzer and Peterson 1991) or nonselective (Den Hertog et al. 1999) destruction of the adrenal cortex with o,p'-DDD (mitotane). Some treatment schedules aim at selective (partial) destruction of the ZF and ZR, sparing the ZG. However, in 5-6% of dogs in which this is attempted, the ZG is also destroyed to such an extent that iatrogenic

hypoadrenocorticism develops. On the other hand, in more than half of the cases in which selective destruction is the aim, there are one or more relapses of hypercortisolism during treatment. In order to avoid these complications, a treatment schedule with the aim of nonselective destruction (complete) of the adrenal cortices and substitution for the induced hypoadrenocorticism (Rijnberk and Belshaw 1988) was developed. This nonselective destruction has been reported to result in fewer recurrences than does selective destruction (Den Hertog et al. 1999).

The induction dose of the selective protocol is 25 to 50 mg/kg per day. After the induction phase of approximately 7 days is completed, the patient is switched to a life-long maintenance dosage, which typically is whatever the induction dose was divided throughout the week rather than daily. When attempting medical nonselective protocol, mitotane should be administered at 50 to 75 mg/kg/d for 25 days. The dose should be divided into at least two administrations throughout the day and administered with food, because is a fat-soluble drug and should not be administrated when the patient is or becomes anorectic. On the third day, hormone supplementation with cortisone at a dosage of 2 mg/kg/d, fludrocortisones at a dosage of 0.0125 mg/kg/d, and sodium chloride supplemented at 1 g/kg/d is usually initiated (Peterson 2007).

The owners were also given injectable glucocorticoid and mineralicorticoid preparations to have on hand, should they miss more than two doses of the oral supplements.

However, o,p'-DDD is no longer regularly used for treatment of PDH, but rather for treatment of inoperable and/or metastasized ATs, with the aim of complete destruction of all adrenocortical cells, including metastases (Kintzer and Peterson 1994). Failure of this treatment is usually ascribed to misdiagnosis of hyperadrenocorticism, improper administration of the drug (for example dosing on an empty stomach), use of inactive drug, or concomitant administration of anticonvulsants (Watson et al. 1987, Nichols et al. 1990).

About ten years ago, trilostane was reported to be a safe and effective alternative for o,p'- DDD in dogs with PDH (Neiger et al. 2002, Ruckstuhl et al. 2002). Trilostane is a competitive inhibitor of

3-β-hydroxysteroid-dehydrogenase enzyme system (HSD3B), an essential enzyme system for the synthesis of cortisol, ALD, and androstenedione (Potts et al. 1978). In dogs with PDH, treatment with trilostane has the potential to significantly decrease basal and ACTH-stimulated plasma cortisol concentration (Neiger et al. 2002, Ruckstuhl et al. 2002, Wenger et al. 2004). This results in loss of negative feedback and thus increased plasma ACTH concentration (Witt and Niger 2004, Sieber-Ruckstuhl et al. 2006). Consistent with its competitive inhibitory effect on HSD3B, trilostane also causes a slight decrease in plasma ALD concentration in dogs with PDH, although ALD usually remains within the reference range (Ruckstuhl et al. 2002, Wenger et al. 2004). In humans, plasma renin activity rather than the plasma ALD concentration is considered to reflect mineralocorticoid deficiency (Loriaux et al. 2001). No detailed information is available on the effects of trilostane treatment on the RAS in the dog. Trilostane is absorbed rapidly from the gastrointestinal tract. Administration with food significantly increases the rate and extent of absorption. A consensus meeting (Amsterdam, April 19th, 2006) recommended a dosage of 2.5 mg/kg per os, once daily. Otherwhise, there is marked variation in the optimal dose and to avoid adverse effects due to overdosage, treatment is started at lower oral dose of 2 mg/kg body weight twice a day. Only capsules of 10, 30 and 60 mg are commercially available, which can be problematic for small dogs. Reformulating the capsules (when country legislation allows it) is an option in small dogs to allow more optimal dosaging. Close monitoring of trilostane therapy is required with the first control visit at 7-14 days after initiation of therapy. The effectiveness of trilostane therapy is judged by resolution of clinical signs associated with glucocorticoid excess and results of an ACTH stimulation test (Neiger et al. 2002, Ruckstuhl et al. 2002). Within about a week on an appropriate dose of trilostane, there is a clear reduction in water intake, urine output, and appetite, followed by improvement in the coat and skin, reduction of central obesity, and increased physical activity (Neiger et al. 2002, Ruckstuhl et al. 2002). Dermatological improvement usually occured later. Dosage adjustments are based also on adrenal reserve tests. The ACTH stimulation

test is best to be performed 2-3 hours after trilostane administration. Indeed, the effects of trilostane last for only a few hours. Once the goal of therapy is achieved (good clinical control with post ACTH cortisol level between 40 and 150 nmol/L), check up is planned every 3 months. Noteworthy are the ultrasonographic changes of adrenal cortex (increased thickness and echogenicity) reported during long term trilostane therapy. Disadvantages to perform an ACTH stimulation test as a monitoring test are that: (1) it only provides information about suppression of cortisol production during a short interval, (2) it is invasive, and (3) the post-ACTH cortisol concentration thought to indicate optimal dosage of trilostane is still arbitrary (Galac et al. 2010). Another way to evaluate trilostane therapy would seem to be measurement of the UCCR. This has been used to detect persisting cortisol secretion after hypophysectomy or nonselective destruction of the adrenal cortex by mitotane therapy (Meij et al. 1998, Den Hertog et al. 1999). Because the UCCR is an integrated measure of glucocorticoid production (Stolp et al. 1983), it might be a more appropriate indicator of the therapeutic efficacy of trilostane than an ACTH stimulation test. The possible side effects of trilostano therapy include vomiting, diarrhoea and lethargy. These can be self-limiting or require cessation of therapy. Rarely, hypoadrenocorticism develops and needs to be treated. This can be confirmed by an ACTH stimulation test (absence of increase in cortisol after ACTH administration). Overdosage of trilostane results in cortisol deficiency and sometimes even mineralocorticoid deficiency (Chapman et al. 2004, Ramsey et al. 2008). In addition, necrosis, apoptosis, and hemorrhage in the ZF and ZR may cause life-threatening hypocortisolism (Reusch et al. 2007). If this occurs, trilostane must be stopped immediately and corticosteroid substitution started. In most cases there is sufficient recovery of adrenocortical function within a few weeks and substitution can be stopped (Wenger et al. 2004, Perez-Alenza et al. 2006). The median survival time for dogs with PDH treated with trilostane once daily (662 days), is similar to that for selective adrenocorticolysis with o,p'-DDD (708 days) (Barker et al. 2005). The median survival time of dogs with PDH for treatment with trilostane twice daily (900 days) is comparable to that for the

nonselective adrenocorticolysis with o,p'-DDD (720 days) (Clemente et al. 2007). If there is metastasis of a functional AT or if neither adrenalectomy nor adrenocortical destruction with o,p'-DDD is an option, trilostane therapy can be used as a palliative treatment (Eastwood et al. 2003, Benchekroun et al. 2008). No differences were found between the survival times of dogs with ATs treated with mitotane compared with those treated with twice daily trilostane. The median survival time for animals treated with trilostane was 353 days, whereas it was 102 days for mitotane (Helm et al. 2011). Others authors reported a median survival time of dogs treated with mitotane equal to 15.6 months and 14.0 months for dogs treated with trilostane (Arenas et al. 2014).

Radiation therapy

Up to 10% to 20% of dogs with PDH have macroadenomas, and the severity of neurologic signs correlates with the size of the tumor relative to the size of the cranial vault. Irradiation of the pituitary gland results in significant reductions in neurologic signs. In a study in which 24 dogs with pituitary macroadenomas received a total of 48 Gy, 10 dogs experienced complete resolution of neurologic signs, and 10 achieved partial remission (Théon and Feldman 1998). The overall median progression-free survival time was 13 months; however, patients with only mild or moderate neurologic deficits had a median progression-free survival time of 21 months. Patients in which the relative tumor size was 12% or less of the cranial vault volume were more likely to achieve complete remission and were four times less likely to experience tumor progression. Endocrine-active tumors were six times less likely to progress. Radiation therapy inconsistently reduces tumor secretion of ACTH, and many patients still require medical management of the Cushing's syndrome (Théon and Feldman 1998). Of dogs that have PDH without accompanying neurologic signs, 50% to 60% have a visible pituitary mass that is identifiable on CT or MRI scans. In two studies, 13 dogs with PDH were imaged with MRI at initial diagnosis and then again 1 year later (Feldman and Nelson 2004; Bertoy et al. 1996). Five dogs did not have a visible tumor on initial imaging; 1 year

later, two of these dogs had visible tumors measuring 6 and 7 mm in diameter. Of the eight dogs with visible tumors on initial imaging, four had tumors that were significantly larger 1 year later. Clinical signs at presentation, the results of endocrinologic testing, and the response to medical management did not predict the tumor size or rate of growth. Because a small relative tumor size strongly predicts the efficacy of radiation therapy, pituitary imaging has been advocated for all patients diagnosed with PDH.

Pheochromocytoma

Pheochromocytomas are rare catecholamine-secreting tumors that most often arise from chromaffin tissue within the adrenal medulla and extra-adrenal sites (sympathetic paraganglioma) (Patnaik et al. 1990). The incidence of pheochromocytoma is 0.5% in human patients with hypertensive symptoms and can be as high as 4% in patients with adrenal incidentalomas described by US (Stein et al. 1991; Mantero et al. 2000). Pheochromocytomas are uncommon in the dog, accounting for 0.01% to 0.1% of all canine tumors, and are often diagnosed at necropsy (Feldman et al. 2004; Gilson et al. 1994). Pheochromocytomas can be solitary or bilateral, benign or malignant, and functional or nonfunctional. Because of the high incidence of tumor invasion into the caudal vena cava and confirmed metastasis at time of necropsy, pheochromocytomas should be considered malignant in dogs (Feldam et al. 2004). In people, approximately 10% of pheochromocytomas are malignant, but in dogs more than half are considered malignant (Barthez et al. 1997; Stenström G et al. 1988; Gilson et al. 1994). These tumors have an unpredictable growth rate and a propensity to invade into surrounding vascular structures, including the phrenico-abdominal vein and caudal vena cava. Local invasion usually involves tumor thrombus extending first into the phrenico-abdominal vein and subsequently into the caudal vena cava from where the thrombus usually extends to varying degrees towards the liver and heart (Lurye et al. 2001; Gilson et al. 1994). Of the various adrenal neoplasms, malignant pheochromocytomas are considered the most aggressive, with direct invasion of adjacent vasculature reported in up to 85% dogs and distant metastasis in up to 40% dogs (Feldman and Nelson 2004; Barthez et al. 1997; Lunn et al. 2013). Immunohistochemical staining for chromogranin A and synaptophysin can be used to distinguish pheochromocytomas from adrenocortical tumors; pheochromocytomas stain positive with high sensitivity and specificity. Up to half of dogs with pheochromocytomas develop concurrent neoplasms (Barthez et al. 1997). Some of these tumors arise from other endocrine tissues, including the pituitary gland, adrenal cortex, thyroid gland, parathyroid gland, and pancreatic beta cells. Multiple endocrine neoplasia

(MEN) syndromes have been well characterized in humans, and similar molecular mechanisms may exist in dogs. Interestingly, though, most concurrent tumors identified are derived from nonendocrine tissues. Among the most commonly identified cancers are soft tissue sarcomas, chemodectomas, hepatobiliary tumors, mammary gland tumors, and osteosarcoma.

Clinical findings

Pheochromocytomas usually are diagnosed in older dogs with a median age of 11 years old. Approximately 50% of pheochromocytomas are discovered incidentally in dogs with no clinical signs or other problem caused by the tumor (Gilson et al. 1994). Clinical signs may be the result of excess catecholamine production or local invasion of surrounding structures. Clinical signs of pheochromocytomas can be vague, intermittent and non specific, including weakness or letargy, polyuria/polydipsia, collapse, panting, anorexia or inappetence, weight lost and vomiting (Barthez et al. 1997). Abnormal physical examination findings include tachyarrhythmias, pale mucous membranes, tachypnea, abdominal pain, fever, a palpable abdominal mass, abdominal distention, dyspnea, and cardiac arrest (Barthez et al. 1997). Hypertension is frequently considered part of the presentation; systolic pressure >160 mm Hg and diastolic pressure >100 mm Hg was found in 43% of dogs affected by pheocromocytoma and among these hypertensive dogs, 70% had a concomitant hyperadrenocorticism (Barthez et al. 1994; Gilson et al. 1999). Hypertension is demonstrated in up to half of all dogs with pheochromocytomas (Barthez et al. 1997). It often is paroxysmal; therefore repeated blood pressure measurements and fundic examinations increase the likelihood of detection. Because of the excess secretion of catecholamines, these tumors often cause debilitating symptoms and a poor quality of life. As to be expected, these symptoms are not always present and certainly do not always constitute a diagnosis.

Diagnosis

Pheochromocytomas are usually first described by diagnostic imaging and then confirmed by a laboratory tests. Abdominal ultrasound scans is described to be very helpful, because many patients are diagnosed with pheochromocytoma only after the discovery of an incidental adrenal mass. Ultrasonography also identifies intravascular invasion with high sensitivity and specificity (Kyles et al. 2003). CT and MRI are the imaging modalities of choice for humans with pheochromocytomas, and early experience with these techniques in canine patients has been encouraging (Rosenstein et al. 2000).

Routine Laboratory

No consistent abnormalities on routine clinical pathology are specific to canine pheochromocytoma. However, these diagnostics are important in identifying concurrent disease. Complete blood cell count is often normal. Most common hemogram abnormalities include anemia, leukocytosis and thrombocytosis. Many dogs showed stress leukogram characterized by neutrophilia, lymphopenia and monocytosis. Packed cell volume and total solids may be elevated because of decreased plasma volume secondary to catecholamine-induced peripheral vasoconstriction, catecholamine stimulated release of erythropoietin by the kidney, or production and secretion of an erythropoietin-like peptide (Maher et al. 1994; Shulkin et al. 1987). Leukocytosis characterized by a mature neutrophilia may also occur and can be the result of catecholamine-induced demargination of neutrophils, necrosis or inflammation of the tumor, or unrelated disease (Maher et al. 1997).

Serum biochemistry panel results are generally unremarkable. Studies reported that most common abnormalities include elevated serum alanine aminotransferase and aspartate aminotransferase activities, serum cholesterol, urea nitrogen and creatinine concentrations; while less frequent biochemical abnormalities described are hyperphosphatemia, hyperglycemia, hypokalemia and hyponatremia. Abnormal urinalysis findings included microscopic hematuria and proteinuria (Barthez et al. 1994; Gilson et al. 1999).

Hormonal Tests

Plasma and 24-hour urine concentrations of catecholamines and their metabolites (metanephrine, normetanephrine, and vanillylmandelic acid) routinely are measured in humans, and elevated concentrations strongly support the diagnosis of pheochromocytoma (Lenders et al. 2002). Epinephrine and norepinephrine are secreted by the normal adrenal medulla. Although dopamine secretion has also been reported, most human pheochromocytomas secrete predominantly norepinephrine or a mixture of norepinephrine and epinephrine (Maher et al. 1994). The excretion patterns of canine pheochromocytomas have not been documented. Catecholamine synthesis in normal and neoplastic adrenal medulla cells is initiated when tyrosine is hydroxylated to dopa. In turn, dopa is decarboxylated to dopamine and transported to the intracellular granules of chromaffin cells. Dopamine is then hydroxylated to norepinephrine; and in some cases, it is further converted to epinephrine (Feldman et al. 2004). The rate-limiting step in catecholamine synthesis is the hydroxylation of tyrosine by the enzyme tyrosine hydroxylase. Norepinephrine normally suppresses catecholamine production through inhibition of tyrosine hydroxylase, but this feedback loop is nonfunctional in pheochromocytomas. This could be caused by increased tyrosine hydroxylase activity or such rapid degradation of norepinephrine that accumulation and subsequent feedback do not occur (Maher et al.1994). Neural impulses mediate secretion of catecholamines by the normal adrenal gland, and excretion occurs by exocytosis of storage granules. Pheochromocytomas are not innervated, and catecholamine release may be initiated by tumor blood flow, direct pressure, or various chemicals and drugs. Catecholamines are converted by monoamine oxidase and catechol O-methyltransferase into inactive metabolites and are excreted by the kidney. These breakdown products include metanephrine (MN) and normetanephrine (NMN), which collectively are referred to as “metanephrines”. These urinary metabolites can be measured to estimate the amount of catecholamine being produced and released by adrenergic or pheochromocytoma tissue (Feldam et al. 2004). In humans various tests may be done to demonstrate excessive catecholamine secretory

capacity by functional phaeochromocytomas, including measurement of urinary catecholamines or their metabolites, and suppression of catecholamine secretion with clonidine or phentolamine (Landsberg et al. 1991). Many human institutions relied upon 24-hour measurements of total urinary catecholamines and metanephrines, because urinary measurements of total catecholamines and metanephrines were found to have a sensitivity and specificity of 98% and 98%, respectively (Sawka et al. 2003; Kudva et al. 2003). Measurement of urinary and plasmatic catecholamines and metanephrines rarely is performed in dogs. Since 24-h urine sampling in dog is impracticable, measurements of urinary fractionated catecholamines and metanephrines was established in spot urine samples by expressing their concentrations as a ratio to the urinary creatinine concentration (Kook et al. 2007). Urinary NMN-to-creatinine ratio has been shown to be significantly increased in dogs with pheochromocytoma compared with dogs with hyperadrenocorticism and healthy dogs (Quante et al. 2010;. Kook et al. 2010), but plasma-free metanephrines previously have only been measured in healthy laboratory Beagles (Francis et al. 2010). More recent studies suggest that the determination of plasma-free metanephrines with high-pressure liquid chromatography [HPLC] provides 100% sensitivity for the detection of a pheochromocytoma compared to only 82% when plasma and urinary catecholamines were determined (Gostelow et al. 2013). Plasma-free NMN concentration is significantly increased in dogs with pheochromocytoma compared with healthy dogs, dogs with nonadrenal disease, and dogs with adrenocortical tumors, and free MN concentration is significantly increased in dogs with pheochromocytoma compared with healthy dogs and dogs with adrenocortical tumors. Plasma-free NMN concentration showed high sensitivity and specificity for diagnosis of pheochromocytoma, whereas plasma-free MN concentration showed moderate sensitivity and high specificity. These results suggest that measurement of plasma-free metanephrines is an effective means of identifying pheochromocytoma in dogs and measurement of normetanephrine is the preferred biochemical test and urine ratio is more accurate to plasma determination (Salesolv et al. 2015).

Diagnostic imaging

Thoracic radiographs could be useful in demonstrating pulmonary metastases and to assess cardiopulmonary structures. The frequency of pulmonary metastases in dogs with pheochroinocytoma is low, but thoracic radiographs should be included in the diagnostic workup of suspected cases (Barthez et al. 1997). Nonspecific cardiovascular changes such as right or left ventricular enlargement have been described, due to intracaval tumor thrombus extending to the right atrium (Gilson et al. 1999). Radiographic evidence of concurrent bronchopneumonia could interfered with the detection of metastases. An abdominal mass may be identified on survey radiographs, with free abdominal fluid radiographically evident. Caudal vena caval angiography could be performed to identify tumor thrombus (Gilson et al. 1999).

Common sonographic characteristics of pheochromocytoma included multicystic and/or multilobular architecture, large size causing displacement of the kidney(s), presence or absence of a capsule and involvement of the caudal vena cava and aorta. It was usually difficult to define limits of the mass relative to the dorsal lumbar muscles and no recognizable adrenal tissue was identified on the affected side (Rosenstein 2000). On abdominal ultrasonography, studies described that intracaval tumor thrombus were correctly identified in 60% of cases, and intra-abdominal metastases in 67%. None of the dogs with pheocromocytoma examined had normal ultrasonographic findings (Gilson et al. 1999). It has been reported that among adrenal tumors, pheochromocytomas is the most variable in size and that no correlation was present between size of the mass, invasiveness and clinical signs in affected dogs and no pattern of echogenicity or architecture was specific for pheochromocytoma (Besso et al. 1997). The advantages of ultrasonography over radiography for adrenal imaging are greater resolution of the adrenal gland for visualization of small masses, identification of retroperitoneal effusion and assessment of adjacent structures (caudal vena cava, aorta and kidneys) for evidence of local invasion. Abdominal

ultrasonography may also be useful for assessment of other organs for evidence of distant metastases.

Computed tomography is useful for canine pheocromocytoma imaging to assess shape, architecture, size of the tumor and to determine margination of a mass and invasion of adjacent structures. Contrast medium is considered to be helpful in humans for assessment of the liver for evidence of metastatic disease and it may be of predictive value for distinguishing benign versus malignant. The use of intravenous contrast medium is controversial in patients suspected of having pheochromocytoma. Warning statements are including in the product insert pamphlet for some contrast media regarding the "risk of a hypertensive crisis following the intravenous administration of contrast media in cases of pheochromocytoma. In several human patients with pheochromocytoma, a marked elevation in blood pressure was noted immediately following intravenous administration of ionic contrast medium (diatrizoate sodium and diatrizoate sodium and meglumine) for selective arteriography and venography. Elevation in plasma norepinephrine was documented in 5 out of 8 humans with pheochromocytoma following intravenous injection of iothalamate meglumine. These observations led to the recommendation to pretreat patients suspected of having a pheochromocytoma with the alpha adrenoreceptor antagonist phenoxybenzamine hydrochloride (Rosenstein 2000). The objective of pretreatment is to prevent a hypertensive crisis due to a sudden catacholamine release by a tumor, prior to contrast radiography or CT. Radioactive iodine-labeled metaiodobenzylguanidine (131I-MIBG) scintigraphy also is commonly used in humans (Norton and Le 2001). MIBG is structurally similar to norepinephrine and is readily taken up by cells of neural crest origin. A pheochromocytoma has been successfully imaged in one dog using 123iodine-labeled MIBG scintigraphy and in two dogs using positron emission tomography (PET) with a fluorinated analog of MIBG (Berry et al. 1993; Berry et al. 2002). Pheochromocytomas may be aspirated with the aid of ultrasonography or CT (Bertazzolo et al. 2014). Cytology might be a simple, minimally invasive method to reach a correct diagnosis.

Cells from these tumors are similar to those from other neuroendocrine tumors, consisting of clusters of fairly uniform cells with numerous naked nuclei. The cytoplasm is light blue and may contain pale basophilic granules. The nuclei are round and have a single, small nucleolus. Significant features of malignancy are not typically present. However, cytology was not reliable in distinguishing benign from malignant neoplasia and additional studies are needed to assess possible risks and complications associated with fine-needle biopsy of adrenal tumors in dogs (Bertazzolo et al. 2014).

Treatment

Surgery is the only definitive treatment for pheochromocytoma (Kyles et al. 2003; Barthez et al. 1997). Hypertension, tachycardia, and arrhythmias are commonly seen during induction of anesthesia and surgical manipulation of the tumor. Dogs should receive phenoxybenzamine, a noncompetitive alpha-adrenergic antagonist, for 1 to 2 weeks before surgery (Kyles et al. 2003; Feldman and Nelson 2004). The standard dosage of phenoxybenzamine is 0.5 mg/kg PO given every 12 hours, but a dosage as high as 2.5 mg/kg every 12 hours may be needed to control hypertension. If the dog is tachycardic, a beta blocker, such as propranolol (0.2 to 1 mg/kg every 8 hours) or atenolol (0.2 to 1 mg/kg PO every 12 to 24 hours), also may be administered. These drugs should be started after alpha-adrenergic blockade has been initiated to prevent unopposed alpha-adrenergic stimulation and severe hypertension. In two studies in which dogs were not pretreated, the overall perioperative mortality was about 40% (Barthez et al. 1997; Gilson et al. 1994). By comparison, when dogs were pretreated, perioperative mortality decreased to less than 20% (Kyles et al. 2003). Dogs without metastatic disease that survive the perioperative period often enjoy long-term survival, although in rare cases metastatic disease can develop several years after surgery (Barthez et al. 1997). Survival after surgery ranged from 1 day to 3.25 years. The prognosis and outcome for dogs that are diagnosed ante-mortem with a pheochromocytoma are usually highly variable due to the unpredictable growth rate and invasive behavior of this neoplasm (Rosenstein

2000). Laparoscopic adrenalectomy in dogs has been successfully performed by several centers with low conversion rates and minimal postoperative morbidity and mortality for adrenocortical tumors (Jimenez Pelaez et al. 2008; Smith et al. 2012; Naan et al. 2013; Mayhew et al. 2015). With careful case selection, laparoscopic adrenalectomy for resection of pheochromocytoma was described to be feasible and could be performed efficiently by experienced laparoscopic surgeons (Pitt et al. 2016). In dogs, it has been reported that laparoscopic adrenalectomy is associated with shorter overall hospitalization times compared to open adrenalectomy. Although postoperative pancreatitis occurred less frequently in dogs in the laparoscopic adrenalectomy group compared to the open adrenalectomy group in a prior study, this difference was not statistically significant (Mayhew et al. 2015). For patients with extensive tumor burdens, surgical debulking, including removal of tumor thrombi from the vena cava, may reduce catecholamine levels enough to control clinical signs. If not, alpha- and beta-adrenergic antagonists may be used in addition.

In humans, chemotherapy protocols, including streptozotocin and combination cyclophosphamide, dacarbazine, and vincristine, have had variable success. Radiation therapy using high-dose 131I-MIBG also has had only moderate success (Norton et al. 2001).

PART 1: ULTRASONOGRAPHIC EVALUATION OF ADRENAL GLAND LESIONS

Chapter 3: Prevalence of ultrasonographic adrenal gland lesions (AGL)

INTRODUCTION:

The use of advanced imaging modalities (eg, ultrasonography, CT, and MRI) in dogs has led to the routine identification of adrenal gland lesions (AGL). Adrenal gland lesions are described as a focal enlargement or masses of the adrenal gland in dogs without prior evidence of adrenal gland disease. The prevalence of such masses in humans is low, affecting only 2% to 5% of patients undergoing CT, but does increase with age (Grumbach et al. 2003; Zeiger et al. 2004; Song et al. 2008). A similar prevalence of 4% (151 out of 3748) was detected by ultrasound in dogs. Adrenal gland lesions may arise from the adrenal cortex or the medulla. Lesions of the cortex can produce excess glucocorticosteroids, resulting in hyperadrenocorticism. Lesions of the medulla can produce excess catecholamines, describing as pheochromocytoma. Primary adrenal neoplasia are rare in dogs and among them, adrenal adenoma, adenocarcinoma, and pheochromocytoma are considered the most common tumors affecting the canine adrenal glands (Lunn et al. 2013; Capen et al. 2002; Myers et al. 1997; Melian et al. 2010). Bilateral adrenocortical enlargement usually occurs secondary to pituitary tumors in dogs, in which case it is due to cortical hyperplasias. Adrenocortical tumors occur in fewer than 50% of dogs with hyperadrenocorticism, with approximately equal numbers of adenomas and adenocarcinomas. Others less frequent lesions have also been reported in dogs, such as adrenal myelolipoma, a benign tumor composed of adipose tissue and hematopoietic cells (Tursi et al. 2005). The goals of the study reported here were to determine the prevalence of AGLs in dogs undergoing routine diagnostic abdominal ultrasonography at a veterinary teaching hospital, to describe the demographics of dogs with AGLs.

MATERIALS AND METHODS:

The study was performed on dogs presented to the Veterinary Teaching Hospital of the University of Turin, between January 2014 and October 2016 for a routine abdominal ultrasonographic

evaluation. For all dogs included in the study a client informed consent was required, according to the requirements outlined by the Animal Care Committee of the Department of Veterinary Sciences of the University of Turin. Patient age, breed, sex and body weight were recorded. Sonographic examinations were performed by the same observer, a competent practitioner with 15 years of training in ultrasonography. A real time ultrasonographic machine (My Lab 70 X-Vision, Esaote Biomedica, Firenze, Italy) with electronic multifrequency linear (frequency range 7,5-12 MHz) and micro convex (frequency range 5,5-6,6 MHz) transducers was used. The operator adapted the settings to obtain the best image quality for the adrenal glands in each dog. The dogs were placed in dorsal recumbency, the ventral abdominal hair was clipped and acoustic gel was applied. All dogs were examined without sedation. Imaging of the adrenal glands was performed by a subcostal approach, placing the transducer on the upper side of the abdomen just medial to the costal arch. Both adrenal glands were scanned in the longitudinal plane. The approach to the left adrenal gland required scanning the area medial to the left kidney and lateral to abdominal aorta, then following the aorta cranially to the origin of the left renal artery, and from this point moving slightly in a cranial lateral direction until a relatively hypoechoic structure could be identified. The left adrenal gland is shaped like a peanut in a ventrodorsal section and the cross section of the phrenic-abdominal vein is visible in the middle ventral portion of the hilum. To identify the right adrenal gland, the approach required scanning the area medial and cranial to the right kidney until the caudal vena cava could be identified on a sagittal plane. The tip of the probe was then tilted in a dorsolateral direction, as previously described in literature (Grooters et al. 1994). The right adrenal gland is a wedge shaped relative hypoechoic structure and the phrenic-abdominal vein crosses the middle portion of the gland longitudinally. The following ultrasonographic parameters were evaluated for each adrenal gland: 1) shape; 2) size (greatest measurement of length (craniocaudal dimension), thickness (ventrodorsal dimension) at cranial, middle and caudal regions of the gland,

made perpendicular to the long axis of the adrenal gland; 3) echogenicity and echostructure; 4) presence of adrenal lesions.

Additional features such as clinical signs, concurrent diseases, endocrinological tests, and final diagnoses were reviewed as appropriate for dogs with an AGL. Statistical analysis to compare population distribution between AGL and normal group was performed by R software (version 3.1.2, R Core Team 2015). The Shapiro-Wilk normality test was used to assess normality of the data. All data were normally distributed and reported as media and ranges. Categorical parameters (sex and breed) were analysed using $\chi 2$ test, while numerical parameter (age and body weight) by t-test. Significance was set at $P < 0.05$.

RESULTS:

The study was performed on 862 dogs presented to the Veterinary Teaching Hospital of the University of Turin, between January 2014 and October 2016 for a routinary abdominal ultrasonographic evaluation. Adrenal glands were not correctly imaged by ultrasound in 75 out of 862 dogs (8.7%) and among them, 29 were left adrenal glands and 46 right adrenal glands. Ultrasonographic examinations showed adrenal gland lesions in 214 out of 787 dogs (27.2%) included in the study. Three hundred seventy-seven adrenal glands were described with ultrasonographic lesions (209 left adrenal glands and 168 right adrenal glands). Adrenal lesions were classified as nodular lesions, masses, homogeneous increase or decrease of volume and heterogeneous echostructure, as summarized in the **Table 1**.

Table 1 Adrenal gland lesions (AGL) found during routinary abdominal ultrasonography

Type of AGL	Left adrenal gland		Right adrenal gland	
	N (n=209)	%	N (n=168)	%
Nodular	97	46.4	82	48.8
Mass-shape	29	13.9	25	14.9
Homogeneous increase of volume	45	21.5	31	18.4
Homogeneous decrease of volume	30	14.3	24	14.2
Heterogeneous echostructure	8	3.8	6	3.6

n= number of adrenal glands

Dogs with AGL included in the study ranged in age from 2 months to 19.3 years, with a median of 8.6 years. Age of dog with adrenal gland lesions (AGL) (median age, 11.2 years; range, 1.2 to 17.2 years) was significantly ($P < 0.05$) higher than dogs with normal glands (median age, 7.5 years; range, 2 months to 17 years). Only 17% of dogs with AGL were < 7 years of age, compared with 65% of the dogs with normal adrenal gland. Median body weight for the AGL group was 18.2 kg (range, 1.4 to 52 kg), which was not significantly different to the normal group (median, 20.3 kg (range, 1.1 to 67.3 kg). Male and female dogs were equally represented in the AGL group (30.1% intact female; 29.3% sterilized female; 27.8% intact male; 12.8% castrated male) and normal group (31.7% intact female; 20.7% sterilized female; 38.2% intact male; 9.4% castrated male). Thirty breeds were represented in the AGL group, most frequently Labrador Retrievers (14%), Shi-Tzu (5.1%), Yorkshire Terriers (5.2%) and West Highland White Terriers (3.7%). Forty-nine mixed-breed dogs had an AGL. Breed distributions in the normal group were the following: Labrador Retrievers (8.2%), German Shepherd (7.9%), Golden Retrievers (5.2%) and American Staffordshire Terriers (5.07%) listed most frequent. Mixed-breed dogs comprised about 36% of the normal group.

Most AGLs were bilateral (63%) and less frequent were unilateral, affecting the left adrenal gland in 31.7% of dogs and the right in 13.5%.

DISCUSSION:

This study described an high prevalence of AGL (27%) reported by routinary abdominal ultrasound. This data could be due to concurrent high prevalence of dogs affected by oncological and endocrinological disease (31.5% and 16%, respectively) presenting in our population, which might bias the population enrolled. Previous study described a prevalence of 4% of adrenal gland lesions discovered incidentally during abdominal ultrasound (Cook et al. 2014), but the inclusion criteria of this study were the absence of any indication of preexisting adrenal gland disease (eg, clinical signs or laboratory findings suggestive of hyperadrenocorticism or unexplained hypertension) or evidence that the adrenal gland lesion was directly related to the decision to perform imaging (eg, signs of acute abdominal pain related to a hemorrhagic adrenal gland mass). Adrenal gland lesions in our study were more likely to be found in older dogs, compared with the normal group, with 83% of affected dogs with more than 7 years of age. Similar results were previously described by literature with a more than 80% of dogs affected by adrenal lesions older than 9 years old (Cook et al. 2014). Results of this study did not suggest any breed or sex predispositions toward an AGL.

A substantial proportion (31.5%) of the dogs with an AGL had concurrent nonadrenal gland neoplastic disease. Therefore, it is possible that some or many dogs of the AGL group with concurrent neoplasia had metastatic adrenal gland lesions. Although metastases to the adrenals are common in humans, they have been described in the 26.7% of adrenal neoplasms in dogs. This anatomopathological study described also that pulmonary, mammary, prostatic, gastric, and pancreatic carcinomas, and melanoma have the highest rates of metastasis to the adrenal glands in dogs (Labelle and de Cock 2005). In our population, among oncological patients there was an higher prevalence of mammary and mastocitoma neoplasmas. Furthermore, results of previous

clinical study indicate that >75% of adrenal gland lesions in dogs with incidental adrenal gland lesions are ultimately proven to be metastatic (Cook et al. 2014). In addition, the true prevalence of concurrent nonadrenal neoplasia could be higher than that reported (31.5%), because many dogs had an open or poorly defined diagnosis at the time of the abdominal ultrasound and an histopathologic diagnosis was available for only 16 of the 214 dogs with an AGL. These is due to the fact that only dogs with larger lesions or those with vascular invasion or distant metastatic lesions may be more likely to be treated with surgery (adrenalectomy) or submitted for postmortem examination with an histopathological definitive diagnosis. Although half of these dogs had a malignant neoplasm (cortical carcinoma [n = 5] or pheochromocytoma [n=3]), this finding was subject to substantial bias and should be interpreted with caution. Potentially, small lesions may be overlooked if overall adrenal gland size and shape are within reference limits or if substantial concurrent disease causes the changes in the adrenal glands to be indiscernible (Besso et al. 1997). More than two-thirds of incidental adrenal gland masses in human patients are benign adenomas with no endocrine activity. Direct comparisons between human and canine studies regarding the risk of malignancy are difficult because of the different biological behavior of adrenal gland medullary tumors (ie, pheochromocytomas) in the 2 species. In humans, these tumors are usually benign and invasion of adjacent structures is not expected (Mittendorf et al. 2007). In contrast, pheochromocytomas in dogs are more likely to be aggressive and vascular invasion and metastases are commonly reported (Gilson et al. 1994). Due to the absence of a definitive diagnosis, further studies are needed to compare the ultrasonographic aspects with the histopathological diagnosis to help the clinician in the differentiation of the AGL.

Chapter 4: Accuracy of Ultrasonographic Measurements of Adrenal Glands

INTRODUCTION

Adrenal gland ultrasonography in dogs has proven a useful clinical screening modality to evaluate adrenal diseases such as pituitary-dependent or adrenal-dependent hyperadrenocorticism, non-functional adrenocortical tumors, adrenal neuro-endocrine tumors, and hypoadrenocorticism (Barthez et al., 1995; Grooters et al., 1995; Grooters et al., 1996; Besso et al., 1997; Hoerauf and Reusch 1999a; Hoerauf and Reusch 1999b; Wenger et al., 2010). Adrenal gland size has been used as a sonographic criterion for differentiating healthy patients from those with pituitary-dependent hyperadrenocorticism (PDH) (Barthez et al., 1995), adrenal-dependent hyperadrenocorticism (ADH) (Benchekroun et al., 2010), and hypoadrenocorticism (Wenger et al., 2010). Barthez et al. (1995) reported 81% sensitivity and 100% specificity for differentiating normal dogs from dogs with PDH at a threshold of 7.4 mm for maximum adrenal gland diameter. Other studies, however, reported that adrenal gland size could be related to patient characteristics such as body weight, breed or age, with a substantial overlap between healthy dogs and those with adrenal gland diseases (Voorhout et al., 1988; Barthez et al., 1995; Grooters et al., 1996; Douglass et al., 1997; Gould et al., 2001; Bertolini et al. 2006; Bertolini et al., 2008; Choi et al., 2008; de Chalus et al., 2013; Mogicato et al., 2011; Soulsby et al., 2014). Recent studies have explored breed-related (Choi et al., 2012) or body weight-related (de Chalus et al., 2013; Soulsby et al., 2014; Bento et al. 2016) factors in order to more accurately describe adrenal gland size and define adrenal gland measurements for each weight class. However, ultrasonography is highly observer- and equipment-dependent (Saunders et al., 1992; Mogicato et al., 2011).

There are many reasons why adrenal gland size vary in dogs. Few studies to date have investigated the association between ultrasound appearance of adrenal glands and their anatomical counterparts. Though these studies have sought to correlate ultrasonographic and post-mortem physical measurements of normal and abnormal canine adrenal glands, the studies themselves are either not

recent or limited by a small sample size (Grooters et al., 1995; Hayles et al., 2010). Moreover, it was found that ultrasound underestimated the actual cranial and caudal maximum diameters and showed only a moderate correlation for the length of the canine adrenal glands. The aim of this study was to determine the diagnostic accuracy of ultrasonography in the evaluation of canine adrenal gland measurements in relation to the actual anatomical adrenal gland size.

MATERIALS AND METHODS

This retrospective cross-sectional study was performed on dogs presented to the Veterinary Teaching Hospital of the University of Turin, between January 2014 and October 2016. All dogs that had undergone abdominal ultrasonographic evaluation, followed within 48 hours by histopathological examination of adrenal glands, were searched in the hospital's electronic databases. Dogs were eligible for inclusion in the study if their clinical records reported ultrasonographic and macroscopic measurements and histological exam results of the adrenal glands. Adrenal glands were collected during adrenalectomy or at necropsy. Dogs that underwent necropsy died of natural causes or were euthanized because of terminal illness. Euthanasia was performed with compassion and dignity after the owners had signed the informed consent form outlined by the Animal Care Committee of the Department of Veterinary Sciences of the University of Turin (in accordance with AVMA guidelines for euthanasia of animals, 2007). Data on the age, breed, and body weight of each dog were collected. Ultrasound examinations were performed by the same sonographer, a competent practitioner with 15 years of training in ultrasonography. A real-time ultrasonographic machine (My Lab 70 X-Vision, Esaote Biomedica, Florence, Italy) with electronic multifrequency linear (frequency range 7.5-12 MHz) and microconvex (frequency range 5.5-6.6 MHz) transducers was used. The operator adapted the settings to obtain the best image quality for visualizing the adrenal glands in each dog. The dogs were placed in dorsal recumbency, the ventral abdominal hair was clipped, and acoustic gel was applied. All dogs were examined

without sedation. Both adrenal glands were scanned in the longitudinal plane using standard techniques, as previously described (Grooters et al., 1994). The following ultrasonographic measurements were taken using the unit's electronic callipers for each adrenal gland on a static image: 1) length (craniocaudal dimension); 2) thickness at cranial (Cran), middle, and caudal (Caud) regions of the gland, perpendicular to the long axis of the adrenal gland; and 3) gland surface area. The adrenal glands were removed and placed in 10% buffered formalin. Seven days later, the formalin fixed tissues were sectioned in the longitudinal plane corresponding to the ultrasonographic section. A picture was taken with a digital camera for each sectioned gland and the sample was then stained with hematoxylin eosin, Grimelius argyrophil, and Gomori trichrome stains for histopathologic examination. The digital pictures of the sectioned glands were measured using Image-Pro Plus software (Media Cybernetics INC, Rockville, MD, USA). Gross measurements were taken in the same dimensions as the previous ultrasonographic measurements both in normal (**Figure 9-10**) and pathological adrenal glands (**Figure 11-12**). A veterinary pathologist reviewed the archived histopathology samples from all 154 adrenal glands. Statistical analysis was performed using readily available software (R software, version 3.1.2, R Core Team 2015). To scale the differences in relation to the gross measurements (gold standard) for each dimension, we divided the absolute error (the difference between the gross and ultrasonographic measurements) by the morphometric value for each observation and expressed it as a percentage (relative error). Normality was assessed using the Shapiro-Wilk test. Given the non-normal distribution of the data, the results are expressed as median values. The presence of statistically significant differences between gross and ultrasonographic measurements was ascertained using Wilcoxon's Signed Rank Test. Comparisons between normal and pathological adrenal glands for measurement errors (both absolute and relative) were assessed using Wilcoxon's Rank Sum test. Correlation between gross and ultrasonographic measurements of all dimensional parameters of the left and right adrenal glands was assessed using Spearman's correlation test (rho) Results with a P-

value of 0.05 or less were considered significant.

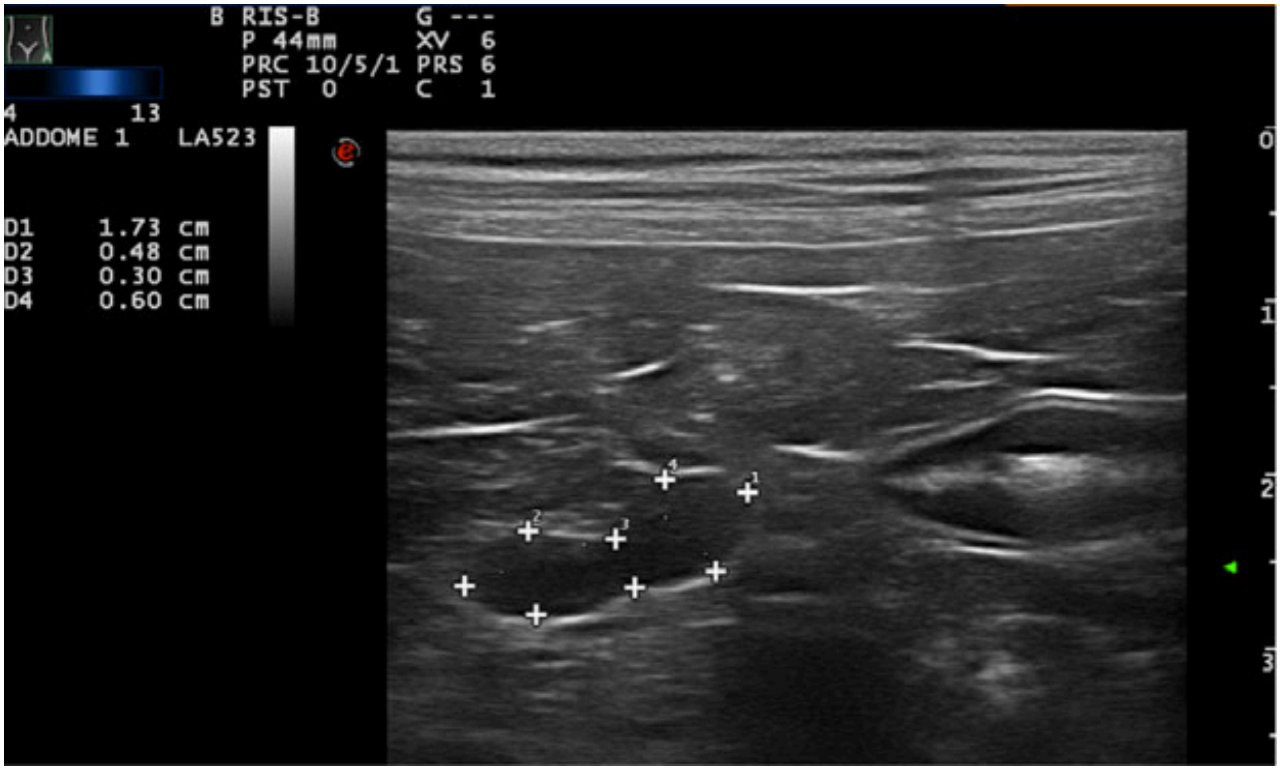

Figure 9: Ultrasound image illustrating measuring the left adrenal gland. Electronic calipers measuring the length and the thickness of the cranial, middle and caudal regions of the adrenal gland displayed in the sagittal plane.

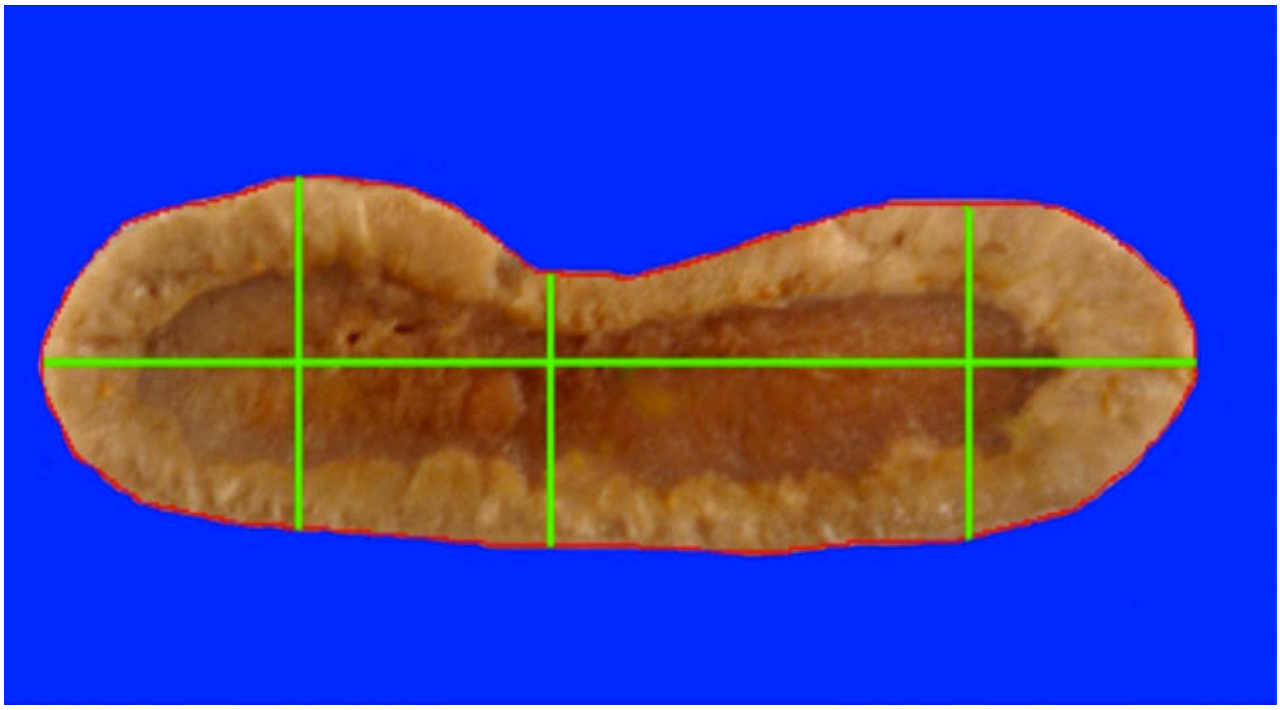

Figure 10: Gross image illustrating the left adrenal gland previously measured by ultrasound. Image-Pro Plus calipers measuring the length and the thickness of the cranial, middle and caudal regions of the adrenal gland sectioned in the same ultrasonographic plane.

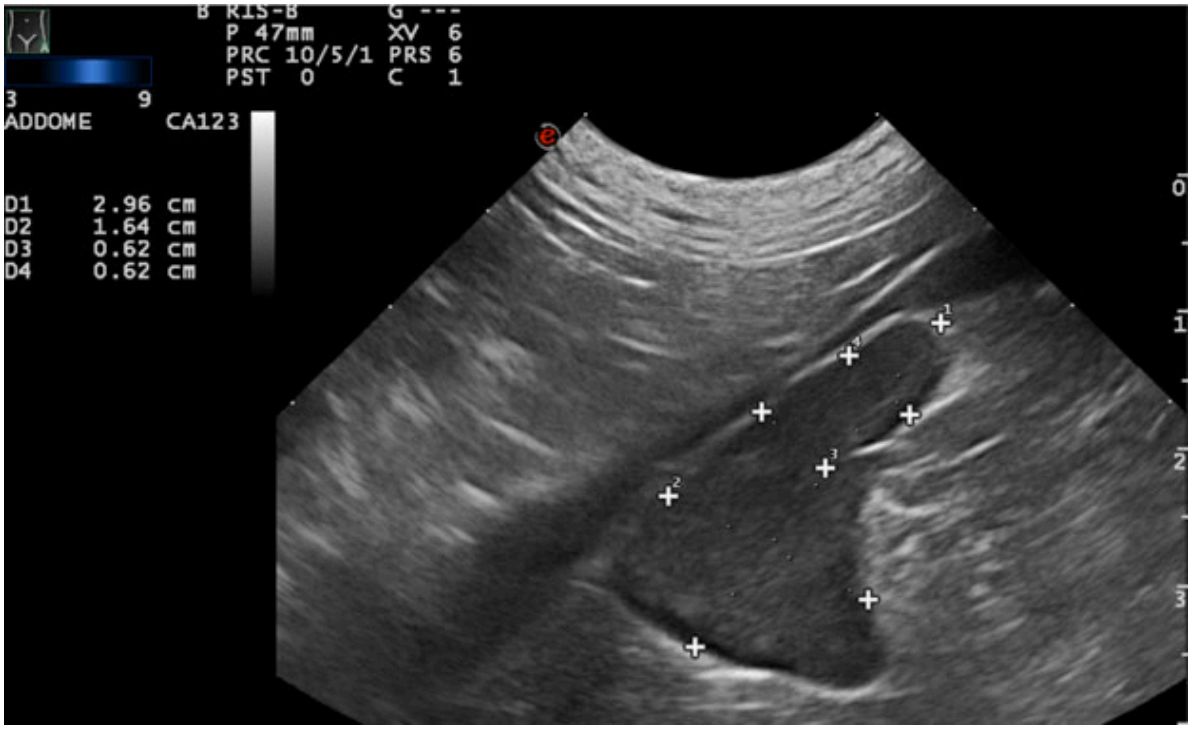

Figure 11: Ultrasound image illustrating measuring the right adrenal gland with a focal enlargement of the cranial pole causing by hyperechoic nodular lesion. Electronic calipers measuring the length and the thickness of the cranial, middle and caudal regions of the adrenal gland displayed in the sagittal plane.

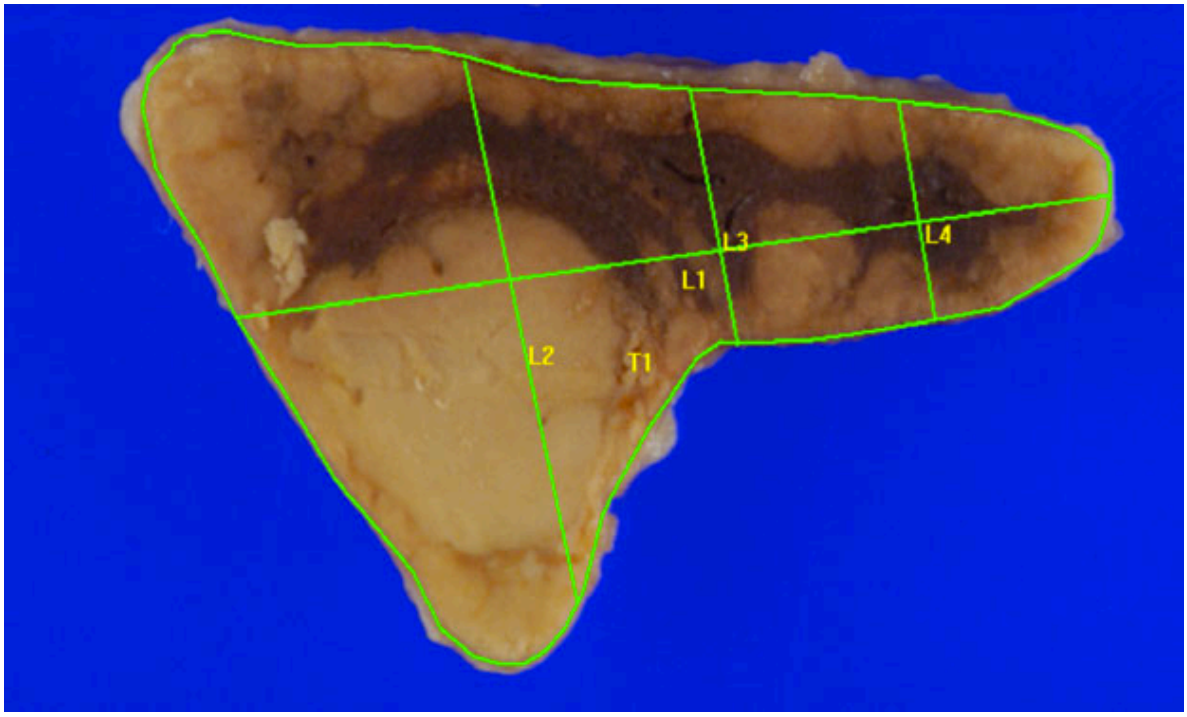

Figure 12: Gross image illustrating the right adrenal gland, with a nodular cortical adenoma in the cranial pole. Image-Pro Plus calipers measuring the length and the thickness of the cranial, middle and caudal regions of the adrenal gland sectioned in the same ultrasonographic plane.

RESULTS

Eighty-five dogs presented to the Veterinary Teaching Hospital, University of Turin, between March 2010 and January 2015, were included in this study and 154 adrenal glands were evaluated (76 right adrenal glands and 78 left adrenal glands). Sixty-nine dogs were euthanized and 16 underwent unilateral adrenalectomy. Because 15 surface area measurements (7 left adrenal glands and 8 right adrenal glands) were missing in the ultrasonographic records, we were unable to compare them with the gross measurements.

The median body weight was 17.5 kg (range, 1.3 to 52 kg) and the median age was 10 years (range, 1 to 17 years). Breeds included: German Shepherd Dog (5), Labrador Retriever (4), Pit bull (4), Border Collie (4), Boxer (4), French Bulldog (3), Cocker Spaniel (3), Yorkshire Terrier (3), Golden Retriever (3), Doberman Pincher (2), Schnauzer (2), Bull Terrier (2), Dachshund (2), Beagle (2) and 1 each of Lagotto, Dalmatian, Doberman, Dogue de Bordeaux, Scottish Airedale, and Hound. Thirty-six dogs were crossbreeds. Of the 154 adrenal glands evaluated, 48 were normal and 106 were pathologic, with nodular hyperplasia in 55 cases, diffuse hyperplasia in 2, accessory tissues in 7, adrenalitis in 4, pheochromocytoma in 11, adrenocortical adenoma in 6, adrenocortical carcinoma in 16, and metastasis in 5. **Tables 1** and **2** present, respectively, the gross and ultrasonographic measurements of the adrenal glands. The differences between gross and sonographic measurements are given in **Table 3**. No statistically significant differences were observed (p-value >0.05) for measurement errors (both absolute and relative) between normal and pathological adrenal glands (**Table 4**).

There was a strong correlation between gross and sonographic measurements of adrenal gland surface area (r_s=0.89, p<0.05 right side and r_s=0.80, p<0.05 left side), length (r_s=0.89, p<0.05 right side and r_s=0.94, p<0.05 left side), and caudal pole thickness (r_s=0.77, p<0.05 right side and r_s=0.68, p<0.05 left side) of both adrenal glands. Also, a strong correlation was noted for the thickness of the cranial pole (r_s=0.62, p<0.05) and the middle part of the right adrenal gland

(r_s=0.70, p<0.05) but a weaker correlation for the left adrenal gland (r=0.46, p<0.05 and r=0.49, p<0.05), respectively.

Table 1: Gross measurements of 154 adrenal glands in dogs.

Right adrenal gland	**Area (mm^2)**	**Length (mm)**	**Thickness Cran* (mm)**	**Thickness middle* (mm)**	**Thickness Caud* (mm)**
Min	30.00	8.80	3.60	3.10	2.60
Median	190.00	25.80	9.50	7.30	5.70
Mean	230.80	25.49	10.38	7.74	6.17
Max	740.00	41.10	29.00	16.30	13.40
n	76	76	76	76	76
Left adrenal gland	**Area (mm^2)**	**Length (mm)**	**Thickness Cran* (mm)**	**Thickness middle* (mm)**	**Thickness Caud* (mm)**
Min	50.00	14.90	3.10	2.10	3.10
Median	160.00	26.40	8.95	5.30	7.20
Mean	240.90	25.41	9.36	5.85	7.43
Max	990.00	46.20	17.80	16.70	15.50
n	78	78	78	78	78

Minimum (min), median, mean, maximum (max), and number of observations (n). * Thickness (ventrodorsal dimension) at cranial, middle, and caudal regions of the gland**.**

Table 2: Ultrasonographic measurements of 154 adrenal glands in dogs.

Right adrenal gland	**Area (mm^2)**	**Length (mm)**	**Thickness Cran* (mm)**	**Thickness middle* (mm)**	**Thickness Caud* (mm)**
Min	70.00	14.90	4.50	2.70	3.20
Median	230.00	27.20	10.95	6.35	5.25
Mean	231.70	27.05	11.47	6.53	5.96
Max	610.00	43.20	25.50	15.30	19.10
n	68	76	76	76	76
Left adrenal gland	**Area (mm^2)**	**Length (mm)**	**Thickness Cran* (mm)**	**Thickness middle* (mm)**	**Thickness Caud* (mm)**
Min	50.00	15.80	2.60	1.20	2.80
Median	140.00	27.15	6.40	4.20	6.30
Mean	164.30	26.36	6.95	4.69	7.17
Max	780.00	46.90	15.90	12.30	20.40
n	71	78	78	78	78

Minimum (min), median, mean, maximum (max), and number of observations (n). * Thickness (ventrodorsal dimension) at cranial, middle, and caudal regions of the gland.

Table 3: Differences between gross and sonographic measurements of 154 adrenal glands expressed as absolute error (A) and relative error (R).

Right adrenal gland	**Area**		**Length**		**Thickness Cran**		**Thickness middle**		**Thickness Caud**	
	R(%)	**A(mm^2)**	**R(%)**	**A(mm)**	**R(%)**	**A(mm)**	**R(%)**	**A(mm)**	**R(%)**	**A(mm)**
Min	-133.30	-130.00	-109.10	-30.35	-135.80	-9.10	-45.59	-3.10	-47.06	-3.50
Median	0.00	0.00	-5.75	-1.50	-6.08	-0.70	14.49	1	3.63	0.20
Mean	-4.37	2.66	-9.23	-2.00	-15.39	-0.93	14.12	1.28	5.06	0.43
Max	50.00	250.00	24.31	5.30	35.80	6.20	50.44	7.30	52.94	6.90
n	68		76		76		76		76	
P-value	1		< 0.01*		< 0.05*		< 0.01*		< 0.01*	
Left adrenal gland	**Area**		**Length**		**Thickness Cran**		**Thickness middle**		**Thickness Caud**	
	R(%)	**A(mm^2)**	**R(%)**	**A(mm)**	**R(%)**	**A(mm)**	**R(%)**	**A(mm)**	**R(%)**	**A(mm)**
Min	-88.89	-90.00	-35.44	-11.20	-94.87	-6.70	-151.90	-5.60	-64.15	-49.00
Median	20.83	40.00	-2.19	-0.55	25.21	2.05	22.22	1.30	3.49	0.20
Mean	16.23	46.32	-3.07	-0.71	19.63	2.46	14.98	1.18	3.02	0,39
Max	51.72	640	22.9	5.50	79.37	10.00	88.12	8.90	55.56	6.00
n	71		78		78		78		78	
P-value	< 0.01*		< 0.01*		< 0.01*		< 0.01*		0.07	

Minimum (min), median, mean, maximum (max), and number of observations (n). To scale the differences for each dimension, we divided the absolute error (A) difference between the gross and ultrasonographic measurements) by the morphometric value for each observation and expressed it as a percentage (R) relative error). Negative values indicate ultrasonographic overestimation.

Table 4: Measurement errors, expressed as absolute error (A) and relative error (R), of 48 normal and 106 pathological adrenal glands.

Right Adrenal Gland	Area		Length		Thickness Cran		Thickness middle		Thickness Caud	
	R(%)	A(mm^2)	R(%)	A(mm)	R(%)	A(mm)	R(%)	A(mm)	R(%)	A(mm)
Normal (n=27) Median (Q1 ; Q3)	0.00 (-0.14 ; 0.06)	0.00 (-0.30 ; 0.20)	-0.05 (-0.11 ; 0.01)	-1.40 (-2.90 ; 0.50)	-0.05 (-0.50 ; 0.06)	-0.40 (-3.45 ; 1.00)	0.08 (0.00 ; 0.24)	0.30 (0.00 ; 2.05)	0.01 (-0.01 ; 0.12)	0.10 (-0.10 ; 1.10)
Pathological (n=49) Median (Q1 ; Q3)	0.00 (-0.11 ; 0.11)	0.00 (-0.20 ; 0.30)	-0.05 (-0.01 ; 0.00)	-1.70 (-3.20 ; 0.20)	-0.08 (-0.29 ; 0.07)	-0.80 (-2.50 ; 0.90)	0.14 (0.00 ; 0.26)	1.00 (0.00 ; 2.30)	0.04 (-0.03 ; 0.15)	0.30 (-0.30 ; 1.10)
No statistically significant differences were observed between normal and pathological adrenal glands (p-value >0.05)										
Left Adrenal Gland	**Area**		**Length**		**Thickness Cran**		**Thickness middle**		**Thickness Caud**	
	R(%)	**A(mm^2)**	**R(%)**	**A(mm)**	**R(%)**	**A(mm)**	**R(%)**	**A(mm)**	**R(%)**	**A(mm)**
Normal (n=21) Median (Q1 ; Q3)	0.18 (0.07 ; 0.30)	0.20 (0.10 ; 0.60)	0.00 (-0.05 ; 0.02)	-0.10 (-1.40 ; 0.80)	0.11 (-0.02 ; 0.34)	0.90 (-0.20 ; 2.70)	0.17 (0.03 ; 0.27)	0.60 (0.10 ; 2.00)	0.00 (-0.05 ; 0.16)	0.00 (-0.30 ; 1.10)
Pathological (n=57) Median (Q1 ; Q3)	0.20 (0.00 ; 0.35)	0.35 (0.00 ; 0.63)	-0.02 (-0.05 ; 0.01)	-0.70 (-1.70 ; 0.30)	0.29 (0.04 ; 0.44)	2.90 (0.30 ; 5.00)	0.22 (0.00 ; 0.40)	1.40 (0.00 ; 2.30)	0.05 (-0.08 ; 0.21)	0.30 (-0.60 ; 1.60)
No statistically significant differences were observed between normal and pathological adrenal glands (p-value >0.05)										

To scale the differences for each dimension, we divided the absolute error (A) difference between the gross and ultrasonographic measurements by the morphometric value for each observation and expressed it as a percentage (R) relative error. Negative values indicate ultrasonographic overestimation. All errors are summarized as median, first quartile (Q1) and third quartile (Q3) since in all tests at least one group (normal or pathological) was not normally distributed.

DISCUSSION

Ultrasonography is a useful imaging modality for evaluating adrenal gland dimension in the diagnostic clinic work-up, but its accuracy needs to be quantified before it can be used as a reliable diagnostic tool. In our sample, the accuracy of ultrasonographic measurements of adrenal glands ranged from 0% to 25.21% (relative error) and the smallest range of error was noted for caudal pole thickness, length, and surface area of the right adrenal gland. Ultrasonographic measurement error of the caudal pole was low (typically 3.64% on the right side and 3.49% on the left side) and showed a strong correlation (r_s=0.77, $p<0.01$ for the right side and r_s=0.68, $p<0.01$ for the left side) with anatomical measurements. Grooters et al. (1995) found a moderate correlation between gross and sonographic measurements of this dimension, and other studies reported that the thickness of the caudal pole of both glands is a much more reliable parameter to distinguish between normal and pathological glands, with less intra- and inter-observer variability (Barberet et al., 2010; de Chalus et al., 2013; Soulsby et al., 2014). For these reasons, the caudal pole appears to be the most reliable and recommended measurement for clinical evaluation, when taking into account the reference range of size for each canine body weight category (Bento et al. 2016). The ultrasonographic measurement error of adrenal gland length was rather negligible, with a small absolute error. These findings are shared by some previous studies (Barthez et al., 1995; Douglass et al., 1997; Mogicato et al., 2011; Soulsby et al., 2014; Wenger et al., 2010) but not by others, which reported that measurement of gland length may vary considerably due to intra- and interobserver factors or physiological parameters (Barberet et al., 2010). Moreover, Grooters et al. (1995) found no correlation with gross measurements, describing it as a less recommendable parameter for practical purposes. Furthermore, length is a measurement obtained perpendicular to the beam direction and it is dependent on the lateral resolution, which is reported to be inferior to the axial resolution used for the other measurements (Bushberg et al. 2011). This concept may explain in part the ultrasonographic error found in the evaluation of length. The parameter with the smallest error

percentage was the surface area of the right adrenal gland, whereas a far greater error percentage (20.83%) was found in measurement of the left adrenal gland. The difference ultrasonographic accuracy between measurement of the right and left surface areas could be due to the different capability of standardized visualization and measurement of the left cranial pole found in our sample. The few previous studies that described a similar dimension of gland surface concluded that gland volume was an inaccurate parameter for estimating the real size of the adrenal glands (Hayles et al., 2010); however, this seems to be the sole exception of adrenal measurements not influenced by animal effect (Bertolini et al. 2006). A larger scale study is needed to determine whether the surface area of the right adrenal gland can be used to distinguish between normal and pathological conditions and give more accurate information about the global aspect of the adrenal glands. The percentage of imaging error of the thickness of the middle part and of the cranial pole of both glands was higher compared to the other parameters. The percentage of measurement error of the thickness of the middle part of the glands was high but the absolute error was low due to the smaller dimension of this portion. The variability in this measurement could be partially due to the vascular overlapping of the phrenicoabdominal vessels with the adrenal cortex, as previously described in literature (Grooters et al. 1995; Barberet et al. 2010); despite the new generation ultrasonographic machines allow for better well-defined anatomical delineation of the vascular structures. Especially for the middle part of right adrenal gland, we found it more difficult to optimize the scan of its long axis and to obtain a good quality of image. The high percentage of measurement error of the cranial pole in both glands was in large part related to the more challenging task of ultrasonographic imaging of this anatomic area. It has been reported that the high variability of adrenal gland measurement is due to the difficulty to identify the cranial pole of the adrenal glands, especially the right adrenal (Barberet et al. 2010; Soulsby et al. 2014). Previous studies have identified several factors that make it more difficult to evaluate the cranial pole and that can result in sonographic measurement error: a) massive presence of adipose tissue, as in patients with large body mass and

high body condition score, which interferes with the contrast between the adrenal glands and the surrounding tissues (Grooters et al., 1994; de Chalus et al., 2013); b) gas present in the gastrointestinal tract can impair correct imaging of adrenal glands, especially on the right side (Grooters et al., 1994; Barberet et al. 2010); c) differences in gland morphology at the level of the cranial pole, generally defined as having a complex shape (Grooters et al. 1995; Barthez et al., 1998). In the present study, cranial pole measurement was more accurate for the right side than the left one. Measurement of the right cranial pole often comprised the thickness of the two lobes forming the gland (**Figure 13-14**), making it difficult to standardize this parameter by ultrasound. The left adrenal gland at the level of the cranial pole is flattened dorsoventrally (Hullinger et al., 2012), with an elliptical cross section (**Figure 15-16**). Also, its position can often vary in relation to vascular landmarks, inducing an oblique orientation of the gland relative to the longitudinal plane (Llabres-Diaz and Dennis, 2003), which can make it difficult to standardize the projection and can explain the high variability in measurement error. The ultrasonographic measurements (median values) generally underestimated the actual size of the adrenal glands, as previously reported elsewhere for adrenal gland thickness, width, and length (Hayles et al. 2010; Grooters et al. 1995). The tendency of ultrasound to overestimate the minimum values may have been due to adrenal gland shrinkage during short-term preservation, which would explain the constancy of this error in all the dimensional parameters we evaluated. Importantly, however, short-term preservation of the adrenal glands was necessary in order to accurately cut the glands in the same plane as the ultrasonographic section. Volumetric shrinkage after formalin fixation has been described (Fox et al. 1985) and could constitute a limitation of the present study. In contrast, the overestimation of the maximum values could have been be due to the difficulty to correctly distinguish the adrenal capsule margins from the surrounding tissues. Not differences in ultrasonographic accuracy were found in the visualization of pathological adrenal glands compared to normal ones. The reliable performance of ultrasound in pathological adrenal glands measurements confirmed that US is a

modality that might help guide the clinical management of dogs diagnosed with adrenal masses (Melian 2012). In conclusion, our results confirm that, for several reasons, the caudal pole of both glands is the best parameter for ultrasonographic evaluation of normal and pathological adrenal gland size in dog. The surface area could be considered as a dimensional parameter for better assessment of the complex shape and the global aspect of the adrenal glands, while standardize ultrasonographic projections are needed to measure the cranial pole of both adrenal glands.

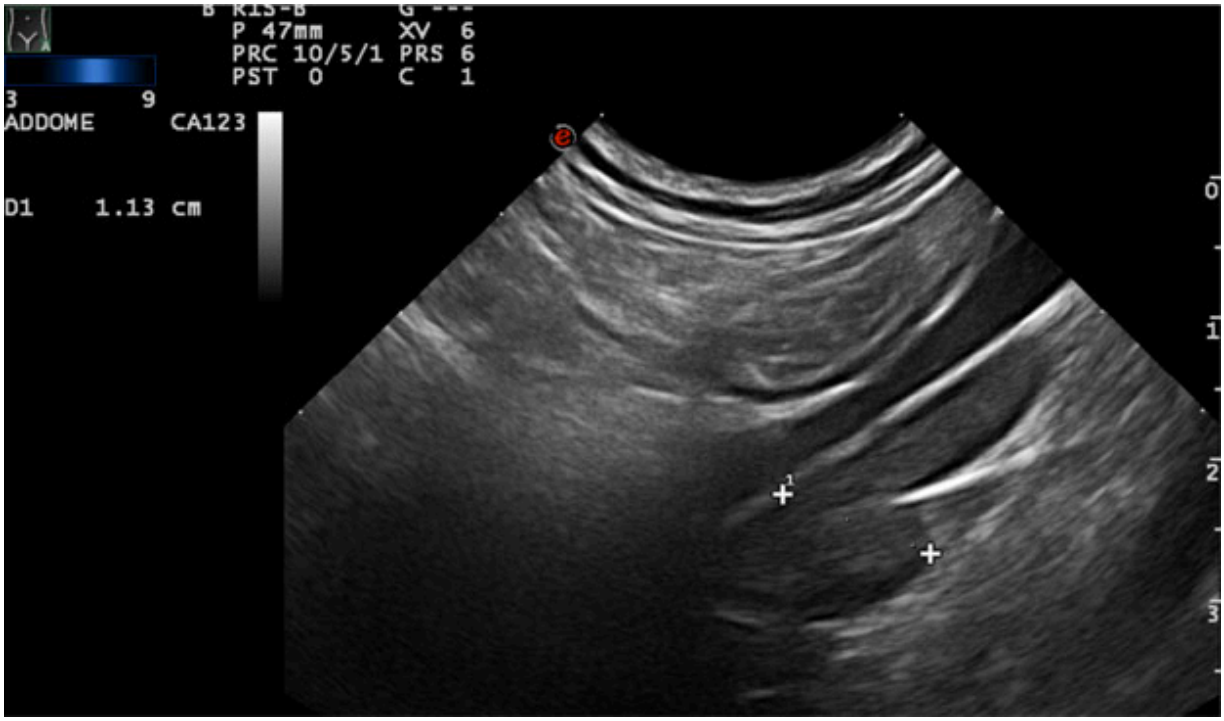

Figure 13: Ultrasonographic image illustrating measuring the right adrenal gland. Electronic calipers measuring the thickness of the cranial pole of the right adrenal gland displayed in the sagittal plane.

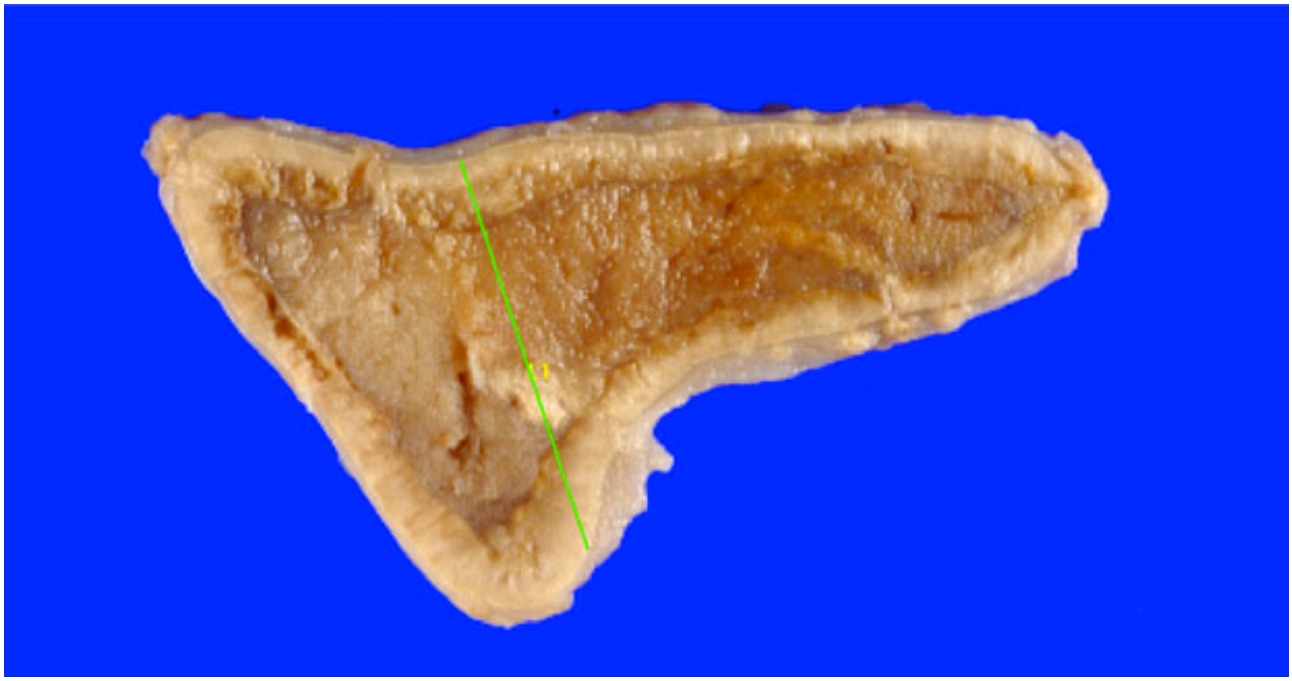

Figure 14: Image-Pro Plus calipers measuring the thickness of the cranial pole of the right adrenal gland sectioned in the same ultrasonographic plane.

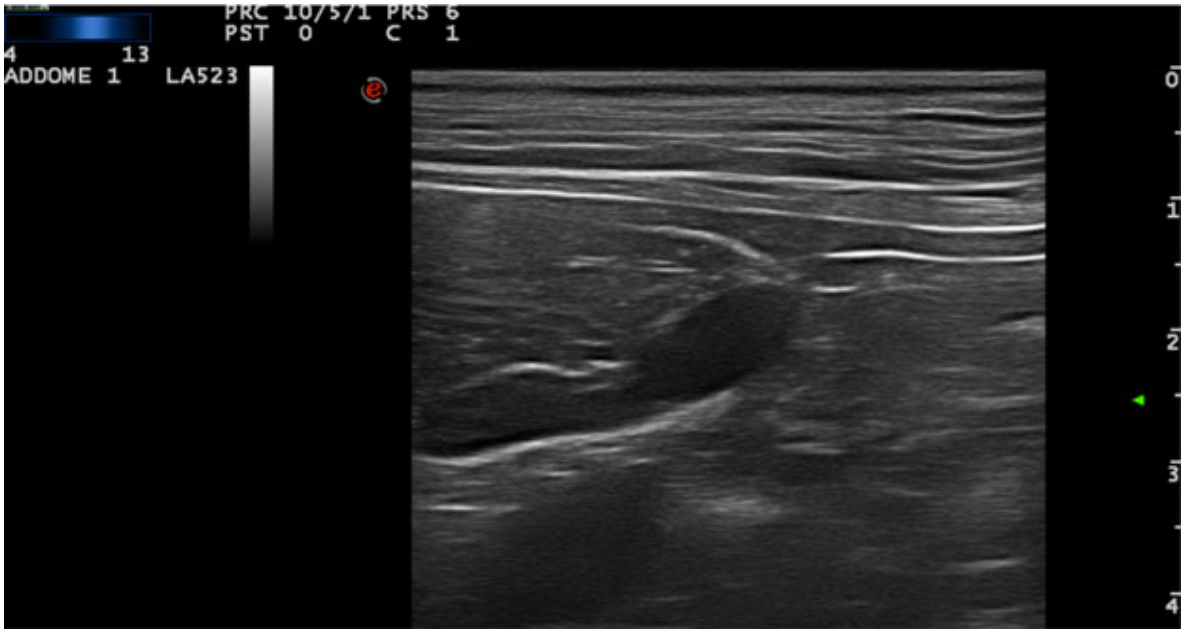

Figure 15: Ultrasound image illustrating the left adrenal gland displayed in the sagittal plane.

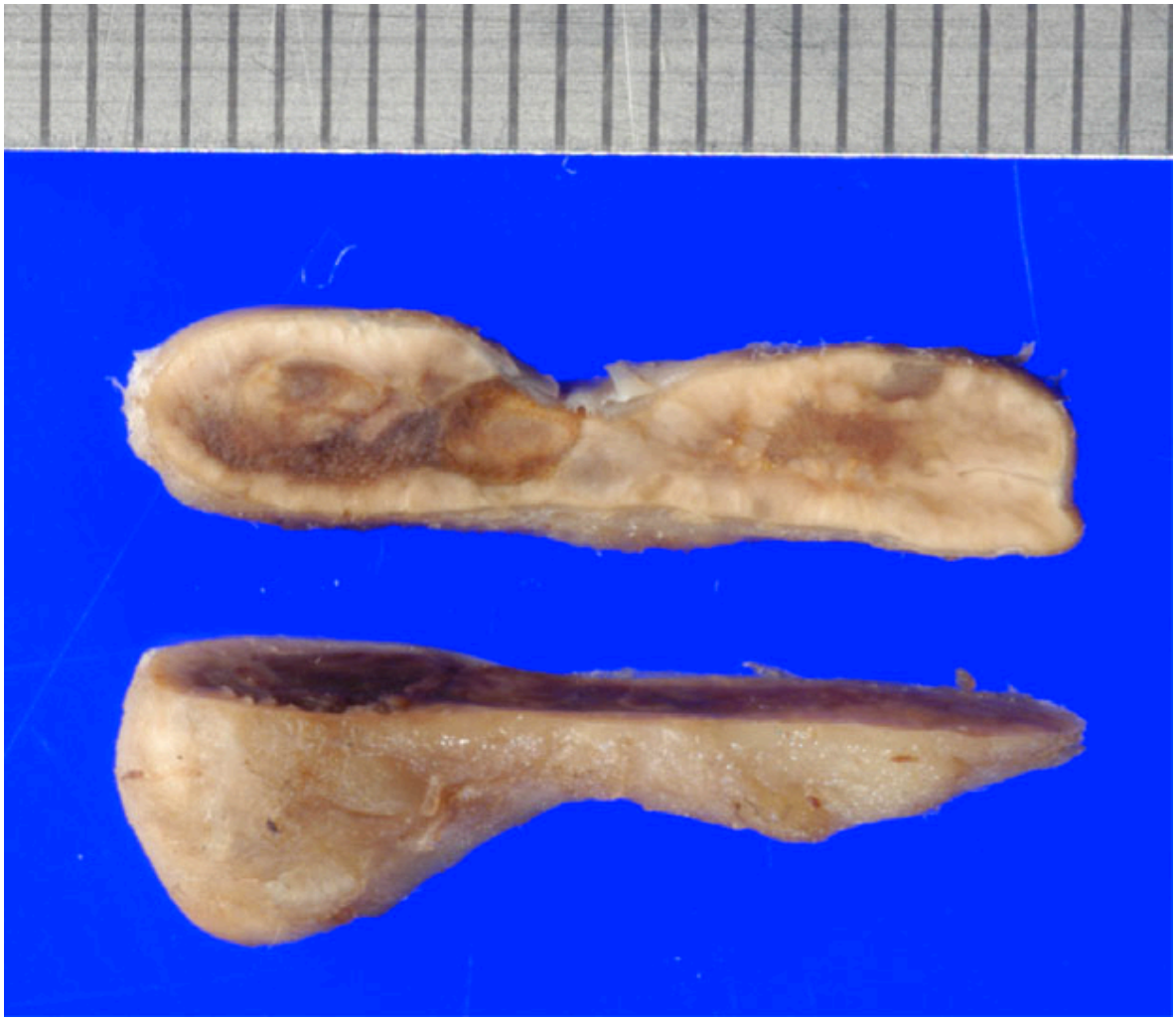

Figure 16: Gross image illustrating the left adrenal gland sectioned in the same ultrasonographic plane.

Chapter 5: Differential Ultrasonographic Parameters of Adrenal Gland Lesions

INTRODUCTION

In human medicine, the use of advanced imaging techniques (e.g., ultrasound, computed tomography, scintigraphy, and magnetic resonance imaging) has resulted in the increasing identification of incidental adrenal gland lesions (Fan et al. 2014). Among the methods of diagnostic imaging, ultrasound is considered a relatively rapid, non-invasive, inexpensive and reliable modality to evaluate suspected adrenal masses (Benchekroun et al. 2010). Beginning in the mid-1990s, ultrasound imaging has become a widely used diagnostic tool in veterinary medicine. Its application in small animal practice has yielded extensive information on normal adrenal gland appearance in the dog (Shelling 1991; Grooters et al. 1995; Douglass et al. 1997; Barberet et al. 2010; Choi et al. 2011; Mogicato et al. 2010; de Chalus et al. 2013; Soulsby et al. 2014), but it has also led to an increase in the incidental discovery of adrenal masses. Though a common occurrence in diagnostic imaging, adrenal masses can constitute a significant clinical dilemma in the dog (Cook et al. 2014). They may be benign (e.g., hyperplasia, myelolipoma, cortical adenoma) or malignant (e.g., pheochromocytoma, cortical carcinoma, and metastases) (Besso et al. 1997). Adrenal gland lesions (nodules and masses) commonly develop in older dogs, but surgery is indicated in only a small fraction of such cases, usually in malignant and hormone-secreting tumors (Massari et al. 2011). While ultrasonography is an effective method for localizing adrenal lesions, standardized ultrasound criteria to distinguish benign from malignant lesions or functional cortisol-secreting from non-functional tumors are lacking (Besso et al. 1997). In large-scale studies involving human patients, ultrasound morphological and dimensional criteria have proven reliable in differentiating adrenal lesions: tumor malignancy potential estimated on the basis of its dimensions alone showed that about 90% of malignant tumors were >40 mm in diameter (Papierska et al. 2013; Fan et al. 2014). In the dog, only one study to date has compared the ultrasonographic appearance of adrenal lesions with their histopathological characteristics. The study was unable to establish definitive

ultrasound criteria to differentiate benign from malignant lesions owing in part to the small sample size (Besso et al. 1997). In the present study involving a large sample of dogs, we asked which ultrasound characteristics of adrenal lesions could predict for malignancy and how accurate ultrasound diagnosis (specificity and sensitivity) was as compared with histopathological diagnosis.

MATERIALS AND METHODS

For this retrospective study, we reviewed the clinical records of dogs presented to the Veterinary Teaching Hospital, University of Turin (Italy) between January 2014 and October 2016. Dogs for which a definitive histopathological diagnosis was available and that had undergone adrenal gland ultrasonography were included whether or not the manifestation of clinical signs was related to adrenal disease. For all dogs included in the study a client informed consent was required, according to the requirements outlined by the Animal Care Committee of the Department of Veterinary Sciences of the University of Turin, approving this study. Adrenal glands were removed and collected during adrenal glandectomy or at necropsy if the dog was euthanized. The glands were placed in 10% buffered formalin and stained with hematoxylin and eosin, Grimelius argyrophil, and Gomori trichrome stains for histopathological examination. An experienced veterinary pathologist, unaware of the ultrasonographic findings, reviewed the macroscopic and histopathology sections of all adrenal glands. Ultrasonography was performed using a B-mode ultrasonographic scanner (MyLab 70 X Vision machine, Esaote, Florence, Italy), with linear (7.5-12 MHz) and microconvex transducers (5.5-6.6MHz). An experienced examiner carried out the ultrasonographic procedures. During abdominal ultrasonography, both adrenal glands were scanned; if lesions were detected, adrenal gland echostructure, echogenicity, dimension, laterality, number, and adjacent vascular invasion were recorded and evaluated. Lesions were classified as follows: A) homogeneous enlargement defined as normal adrenal gland shape with rounded contour; B) irregular enlargement defined as total loss of normal adrenal shape, echostructure, and

dimension, with a mass aspect; C) nodular lesion defined as a round, well-defined focal parenchymal lesion, without loss of global shape; and D) multiple nodules defined as multiple, well-defined focal parenchymal lesions, without loss of adrenal global shape. Lesion size was determined by measuring the greatest dorsoventral and craniocaudal dimension in a longitudinal plane, as described by Hoerauf et al. (1999) [23]. Conventional gray-scale and color Doppler ultrasound were used to assess the adjacent vascular structures. Statistical analysis to compare the ultrasonographic predictive parameters among benign and malignant lesions was performed by R software (version 3.1.2, R Core Team 2015). The Shapiro-Wilk normality test was used to assess normality of the data. Since data were not normally distributed, they were reported as medians and ranges. Categorical parameters (side and shape) were analysed using $\chi 2$ test and Fisher's exact test, while numerical parameter (size) by Kruskal–Wallis test and Wilcoxon signed-rank test was used as post hoc test. Significance was set at $P < 0.05$. Ultrasound sensitivity and specificity in detecting adrenal lesions were calculated using 2x2 contingency tables. Ultrasound characteristics of each lesion were compared with their corresponding histopathological characteristics. The processing concerning the description of the statistic distributions of observations was achieved by a self-developed routine implemented in the IDL 8.0 programming language (ITT Visual Information Solutions).

RESULTS

The clinical records of 119 dogs that had undergone ultrasound adrenal gland and histological examination were reviewed. Unilateral adrenal glandectomy was performed in 9 dogs and necropsy in 110. Histology was performed on 229 adrenal glands. Histopathological changes were noted in adrenal glands obtained from 69/119 dogs, with bilateral lesions in 36 dogs and unilateral in 33. Adrenal glands were histologically normal in 50/119 dogs. Dogs with adrenal gland lesions, based on histopathological exam, were generally older (median age 10.5 years, range 1-17; median body

weight 17.5 kg, range 2.3-52) than those with normal adrenal glands (median age 5.8 years, range 1.5-12; median body weight 23.1 kg, range 3.5–65) and equally distributed between sexes, 47.5% were male and 52.5% female. Forty-seven different breeds were included. The most commonly represented breeds were mixed breed (32), German Shepherd Dog (7), Labrador Retriever (5), Beagle (5), Boxer (5), Pit bull (4), French Bulldog (3), Bull Terrier (3), Yorkshire Terrier (3), Golden Retriever (3), Doberman Pincher (3), Schnauzer (3), Border Collie (2), Dogue de Bordeaux (2), Dachshund (2), Cocker Spaniel (2), Dalmatian (2), and Doberman (2). Other less represented breeds (31) were found. Age, sex, weight and breed were not significantly different between dogs with normal adrenal glands and pathological ones and between malignant and benign lesions.

Histological diagnosis revealed cortical hyperplasia (n=67), adenocarcinoma (n=17), pheochromocytoma (n=10), metastases (n=7), adenoma (n=4), and other minor lesions (adrenalitis) (n=4). The prevalence of adrenal neoplasms was 18%, of which 82.2% were primary adrenal tumors and 17.8% metastatic lesions. Ultrasound imaging to detect adrenal lesions had high specificity (100%) but low sensitivity (63.7%). Adrenal lesions were not displayed in 56.8% (62/109) of cases: 67.7% (47/62) as cortical hyperplasia, 9.6% (6/62) as cortical adenocarcinoma, 6.4% (4/62) as metastasis, 4.8% (3/62) as cortical adenoma, 1.6% (1/62) as pheochromocytoma, and 1.6% (1/62) as adrenalitis. **Table 5** presents the number, type, and characteristics of adrenal lesions identified at ultrasonography and histopathology. Adrenal carcinoma was the most common type of adrenal primary neoplasia (17/31), and it was generally unilateral, affecting the left and right adrenal gland in 8 (47%) and 9 (53%) dogs, respectively. Adrenal glandectomy was performed in 9 dogs with histopathologically confirmed unilateral adrenal carcinoma while necroscopy was performed in the other 8 cases, in 4 of which the contralateral gland was found atrophic. The adrenal gland usually maintained its normal shape. In 82.3% (14/17) of cases the tumor presented a nodular shape (single or multiple nodules) (**Figure 17**), of which 76.4% (13/17) showed a lesion >10 mm. Of note, in 2 cases ultrasound overestimated the actual lesion size. The median diameter

was 15 mm (range 3-37). The echotexture was generally heterogeneous, with several small areas of inner calcification producing acoustic shadowing. In the patients with adrenocortical carcinoma, echogenicity ranged from hypo- to hyperechoic, compared to the renal cortex, and it was associated with microscopic findings of focal areas of necrosis and hemorrhage. Evidence of vascular invasion of the phrenicoabdominal vessels was noted in 23.5% (4/17) of dogs with a right-sided tumor. No invasion of the caudal vena cava or abdominal aorta was noted. Ultrasonography failed to detect lesions in 6 cases: lesions were <3 mm in 4 cases and <20 mm in 2 cases. Pheochromocytoma was the second most frequent primary adrenal neoplasia (10/31). In 5 cases a large, amorphous encapsulated mass, with irregular margins and loss of normal shape and parenchymal structure, was visualized (**Figure 18**). The remaining lesions were solitary or multiple nodules with normal adrenal shape. In 6/ 10 cases, pheochromocytoma was >10 mm in diameter and in 3 cases ultrasound overestimated the actual lesion size. The median diameter was 13 mm (range 7-62). No specificity in echogenicity was noted. Tumor thrombus extending into the phrenicoabdominal vein and caudal vena cava was visualized in 40% (4/10) of cases. In 1 case, both adrenal glands were affected by a pheochromocytoma caudally displacing the kidneys, with distortion of normal renal shape. No distant metastases were found in any of these 10 dogs. Ultrasound failed to detect pheochromocytoma in only 1 case and no gross lesions were detected at necroscopy. Cortical adenoma was a rare adrenal primary neoplasia (4/31), presenting as solitary or multiple nodular lesions affecting both adrenal glands (median diameter 5.5 mm, range 4-15). The only one visualized on ultrasound appeared as heterogeneous multifocal nodules throughout the parenchymal tissue, with several small areas of inner calcification (**Figure 19**). Compared with adrenocortical adenomas, the nodules in cortical hyperplasia were smaller and more numerous. Adrenocortical hyperplasia (67 cases) affected both adrenal glands in 32/35 (91%) cases. All adrenal glands affected by cortical hyperplasia maintained their normal shape. In 97% of the cases they were <10 mm and in 55% of them they had a multinodular aspect affecting the entire glandular parenchyma.

Ultrasound detected cortical hyperplasia in 30% of cases; hyperplasia was characterized by focal nodules ranging from 3.1 to 10 mm in diameter (**Figure 20**). Adrenal metastasis was usually bilateral and presented as multifocal and heterogeneous nodules with irregular margins (**Figure 21**). The adrenal metastases stemmed from 2 primary neoplasms: splenic hemangiosarcoma with bilateral lesions in 3 dogs and lung carcinoma in 1 dog. All metastatic lesions were <10 mm in diameter. A comparison between the ultrasonographic predictive parameters for the diagnosis of benign (cortical hyperplasia and cortical adenoma) and malignant (cortical carcinoma, pheochromocytoma, metastasis) lesions showed significant statistical differences ($P<0.05$) for all the parameters evaluated (lesion side, shape and size) (**Table 6**). Furthermore, a statistically significant association was noted between ultrasonographic nodular shape and the presence of benign lesions, such as cortical hyperplasia and cortical adenoma ($P=0.006$), whereas an irregular enlargement of the adrenal glands was significantly associated with malignant neoplasia, such as pheochromocytoma and cortical carcinoma ($P=0.004$). Bilaterality of adrenal lesions was significantly associated with benign lesions, such as cortical hyperplasia ($P=0.04$) and right adrenal lesions with malignant lesions ($P=0.03$).

Table 5: Ultrasonographic and histopathological features of adrenal gland lesions.

Histopathological Diagnosis		No. of Lesions	Adrenal Side			Lesion Shape				Lesion Size (mm)			
			L	R	Bi	A	B	C	D	<3	3.1-10	10.1-20	>20.1
Cortical Hyperplasia	US	20	6	4	10	3		17			15	5	
	AP	67	1	2	64	1	1	23	42	34	31	2	
Cortical Adenoma	US	1	1						1			1	
	AP	4	2	2				2	2		3	1	
Cortical Carcinoma	US	11	5	6		1	3	6	1			5	6
	AP	17	8	9		1	2	12	2		4	9	4
Pheochromocytoma	US	9	2	5	2		5	2	2			3	6
	AP	10	3	5	2		5	2	3		4	2	4
Metastasis	US	3		1	2			1	2	2	1		
	AP	7		1	6				7	6	1		

US: Ultrasonographic findings; AP: histopathological findings; L: left side; R: right side; Bi: bilateral side. A Homogeneous enlargement; B Irregular enlargement; C Nodular lesion; D Multiple nodular lesions. Minor lesions (4 adrenalitis) were not express in the table because they were not detect by both ultrasonographic and macroscopic evaluations.

Table 6: Ultrasonographic predictive parameters for the diagnosis of benign and malignant adrenal lesions.

Histopathological Diagnosis	No. of Lesions	Adrenal Side			Lesion Shape				Lesion Size
		n (%)			n (%)				Median (Range) mm
		L	R	Bi	A	B	C	D	
Benign Lesions	21	7 (50)	4 (25)	10 (71.4)	3 (75)	0 (0)	17 (65.4)	1 (16.7)	7.05 (3-16.1)
Malignant Lesions	23	7 (50)	12 (75)	4 (28.6)	1 (25)	8 (100)	9 (34.9)	5 (83.3)	10.8 (3-70.5)
p-value		1	0.03*	0.04*	0.33	0.004*	0.006*	0.18	0.0001*

L: left side; R: right side; Bi: bilateral side. A Homogeneous enlargement; B Irregular enlargement; C Nodular lesion; D Multiple nodular lesions. *P <0.05.

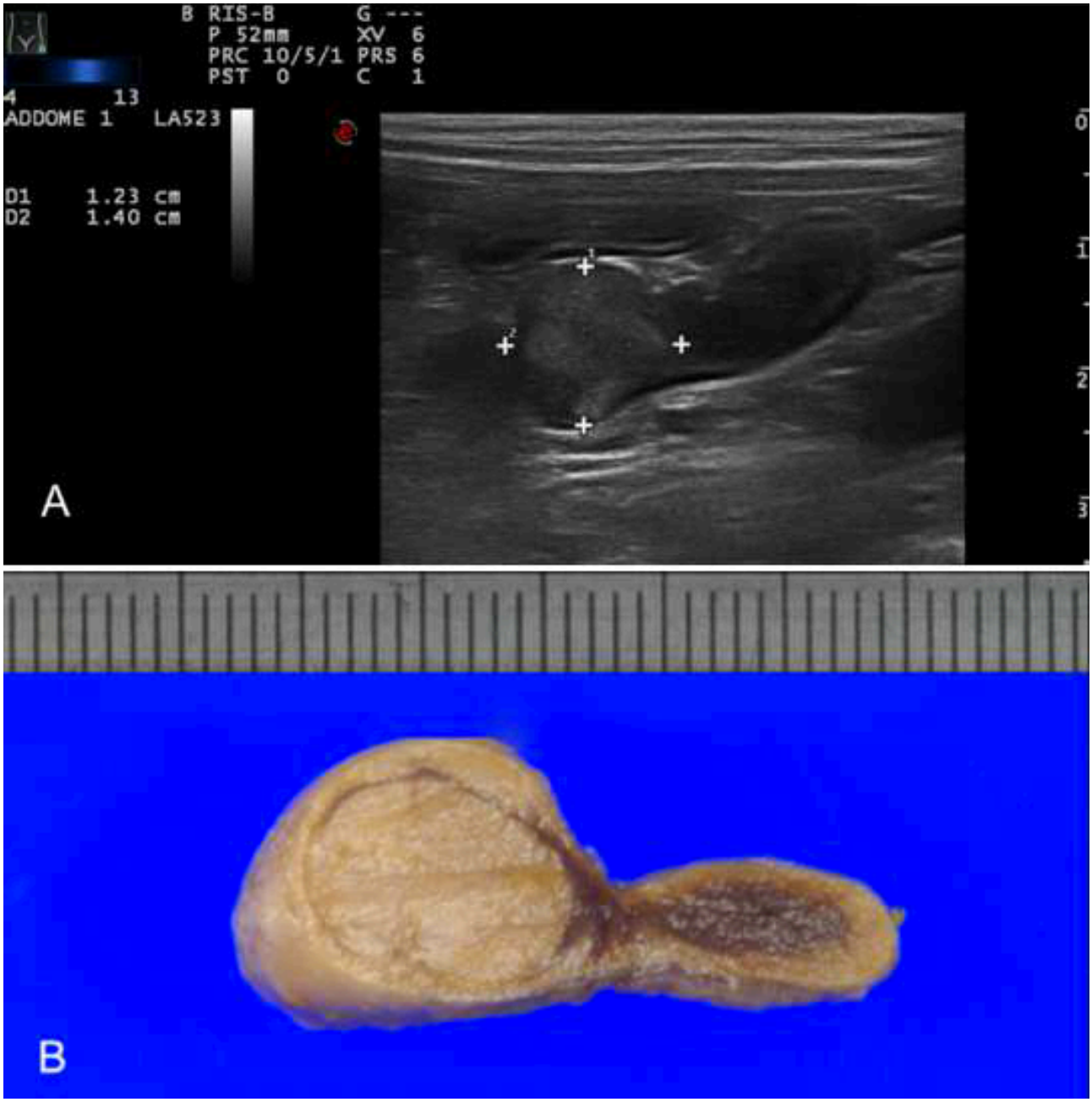

Figure 17 CORTICAL CARCINOMA: A) ultrasonographic and B) macroscopic images of left adrenal cortical carcinoma nodular lesion of, illustrating a left adrenal nodular lesion, 12 x14 mm size, middle uniform echogenicity, and well defined margins.

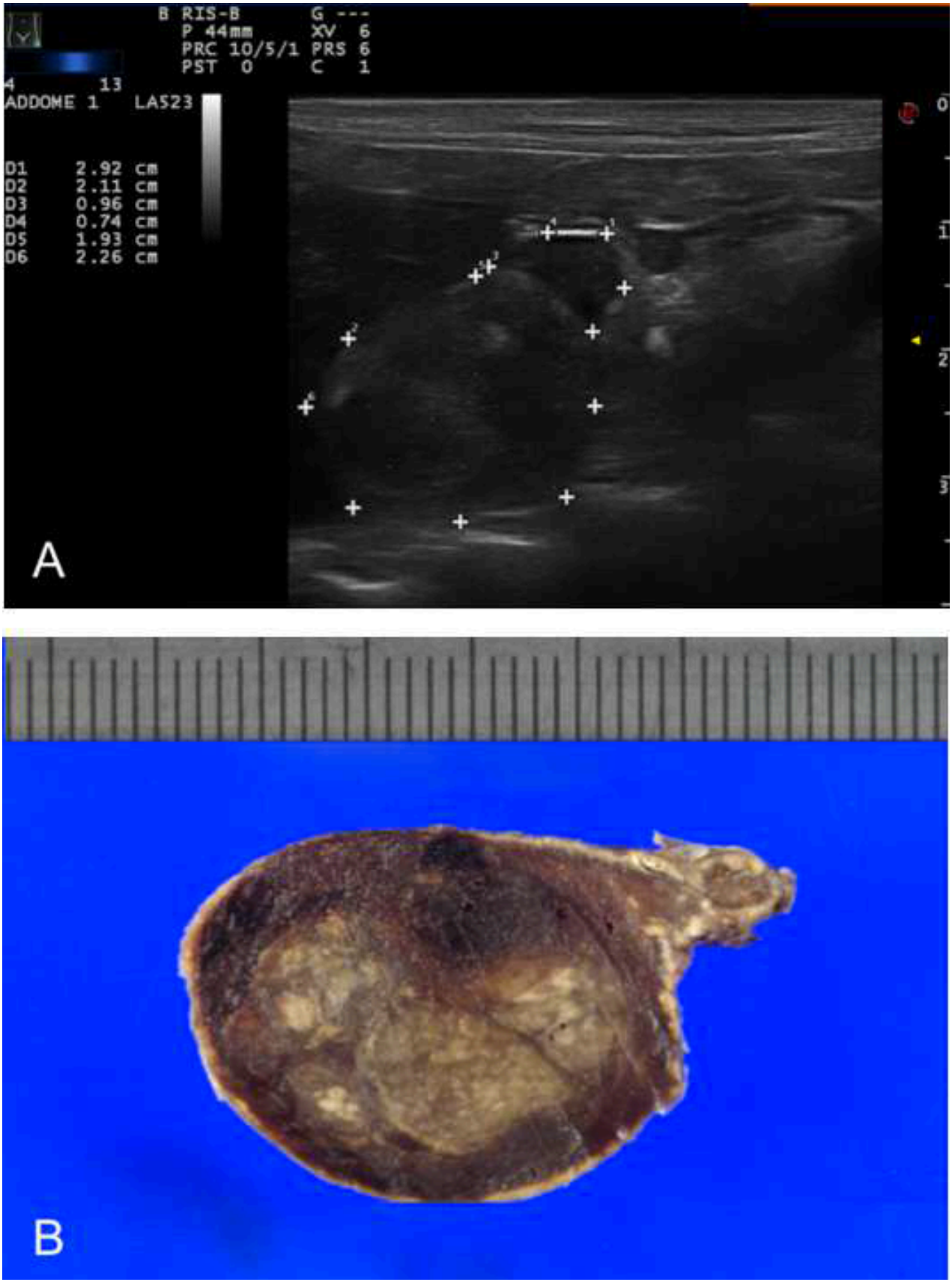

Figure 18 PHEOCHROMOCYTOMA: A) ultrasonographic and B) macroscopic images of right adrenal mass pheochromocytoma of, 29 x 21 mm size, heterogenic echotexture and irregular margins.

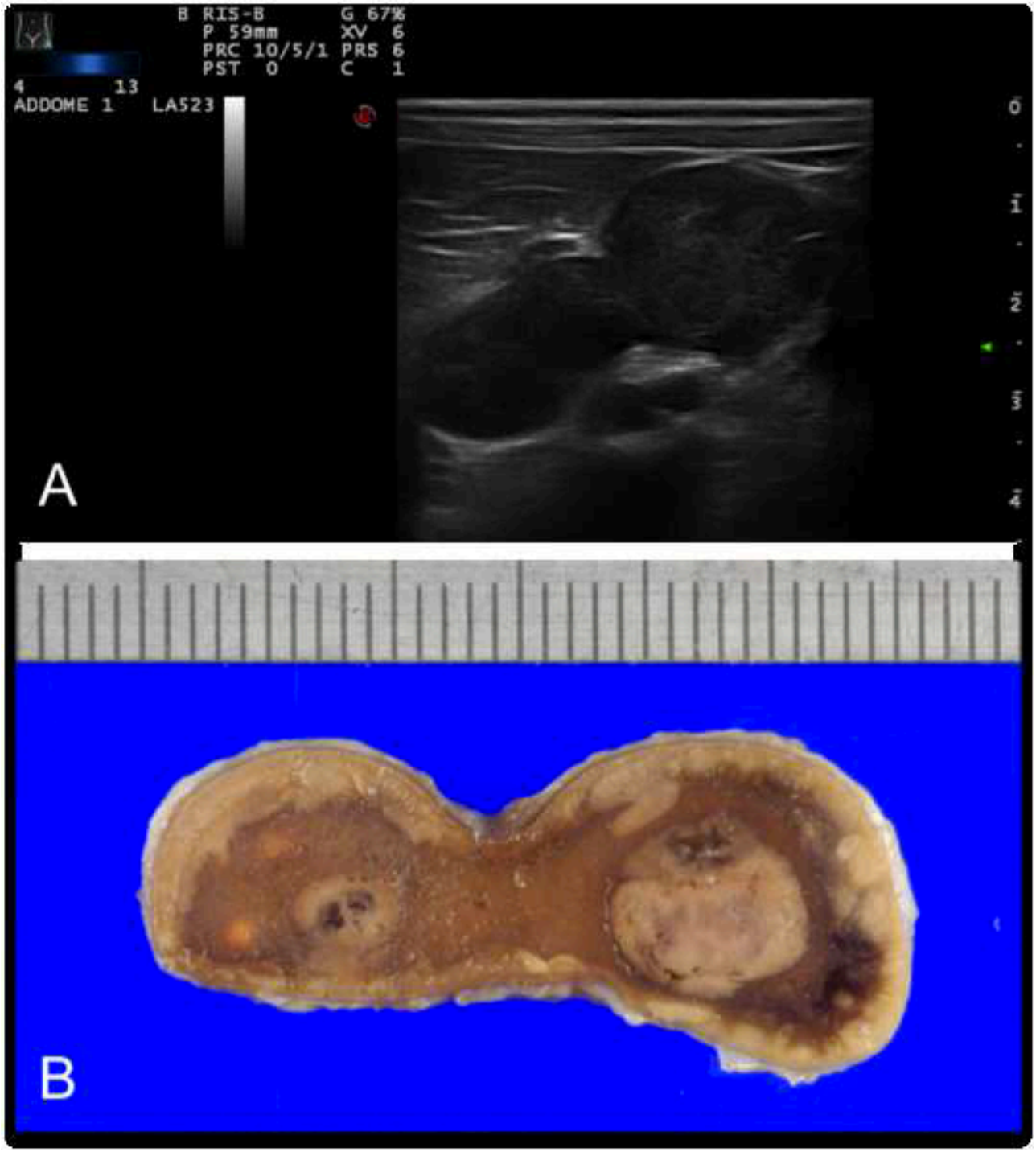

Figure 19 CORTICAL ADENOMA: A) ultrasonographic and B) macroscopic images of left adrenal gland cortical adenoma showing with heterogeneous multifocal nodules with small areas of necrosis, calcification, and hemorrhaging.

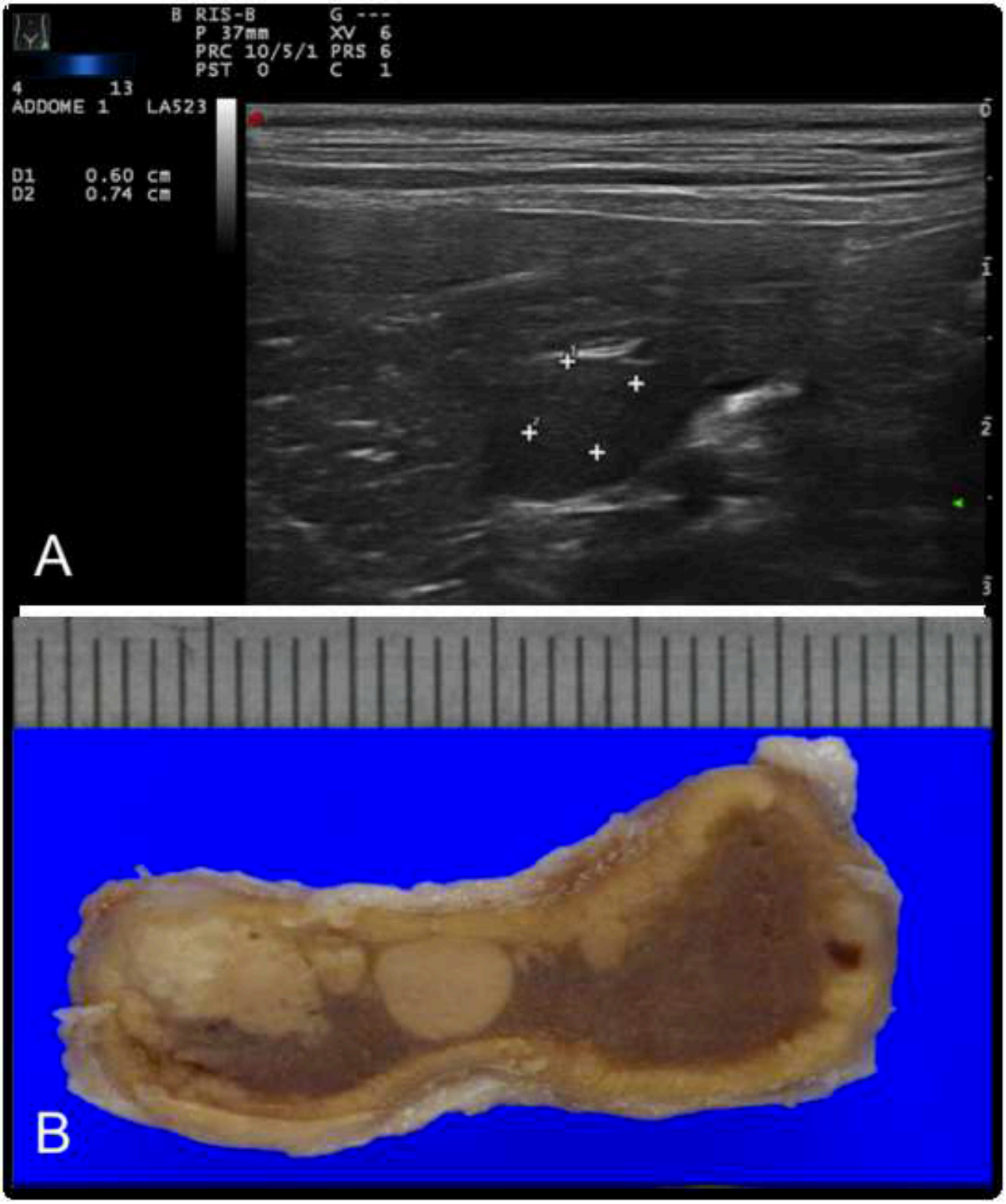

Figure 20 CORTICAL HYPERPLASIA: A) ultrasonographic and B) macroscopic images of left adrenal gland cortical hyperplasia with multinodular aggregated lesions.

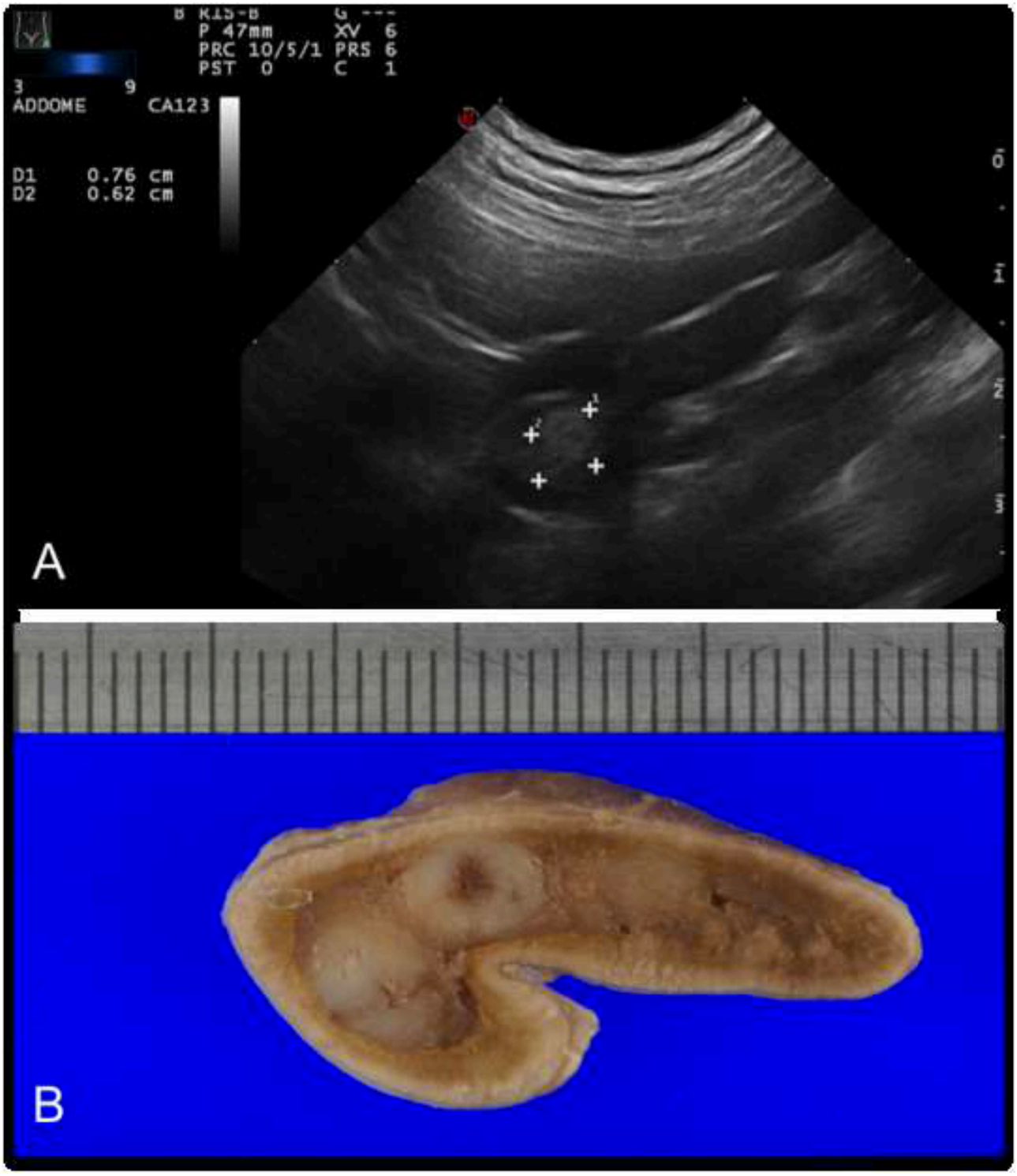

Figure 21 METASTASES: A) ultrasonographic and B) macroscopic images showing a right adrenal gland lung carcinoma metastases with multifocal and heterogenic nodular lesions with irregular margins.

DISCUSSION

The prevalence of adrenal gland neoplasia in our study was 18.1%, of which 81.7% were primary adrenal neoplasia and 18.3% metastatic lesions. The most frequent neoplastic type was adrenocortical carcinoma (44.7%), followed by pheochromocytoma (26.3%), metastases (18.4%), and adrenocortical adenoma (10.5%). A high prevalence (32%) of adrenal cortical hyperplasia was also noted. As reported in humans, the majority of histologically diagnosed adrenal lesions were benign, such as cortical hyperplasia (65.1% of cases in our series) rather than neoplastic (Fan et al. 2014). Our results are in agreement with data reported by a previous study in dogs based on histological examination showing a prevalence of 19% of adrenal neoplasia and 41.5% cortical hyperplasia (Myers et al. 1997). Adrenal lesions are more likely to be found in older dogs (Cook et al. 2014) with a median age of 10.5 years for dogs with adrenal gland lesions and 5.8 years for those with normal adrenal glands. Although no statistically significant differences between age and type of adrenal lesions were found (P=0.20), the incidence of cortical hyperplasia seemed to increase with advancing age, with 67% of the dogs showing cortical hyperplasia being ≥ 8 years old. Chronic or severe illness in old dogs may reflect an increase in adrenocortical demand in response to stress and cause adrenocortical hyperplasia. Adrenocortical hyperplasia is also described to be more frequent in small dogs, but in this study there was no significant correlation between adrenal lesion type and body weight (Cook et al. 2014). In terms of diagnostic tools, ultrasound imaging had high specificity (100%) but low sensitivity (63.7%) in the detection of lesions. However, sensitivity seemed to improve with increasing lesion dimension. Ultrasonography failed to detect lesions <3 mm in diameter in 95% of the cases and lesions between 3 to 10 mm in 46.8% of the cases. Ultrasound failed to detect 67.7% (47/62) of cortical hyperplasia lesions and 5/7 of the metastatic lesions. In contrast, nearly all lesions (20/22) >10 mm were correctly detected. Lesions that appear benign on ultrasound images can be confused with other types of abnormalities, including malignancy. Our data indicate that although structural features, such as lesion size, shape,

laterality, and echotexture, may provide helpful diagnostic ultrasound criteria, such features alone are not necessarily pathognomonic. Of the parameters evaluated, lesion size seemed to be a distinguishing feature, as smaller adrenal lesions associated with a benign outcome whereas larger ones were more likely malignant (P=0.001). Adrenal gland lesion dimension may be a useful ultrasonographic indicator to predict malignancy. Seventy percent (19/27) of adrenal primary tumors were in fact >10 mm, 30% >20 mm, and all adrenal lesions >20 mm in diameter were malignant tumors (pheochromocytoma and carcinoma both in 4 cases each). This finding is consistent with previous reports describing malignant adrenal tumors as lesions >20 mm in diameter (Kantrowitz et al. 1986; Poffenbarger 1988; Bondyad et al. 1987) or 75% between 20 and 40 mm and 100% >40 mm in maximum diameter (Besso et al. 1997). In agreement with this Cook et al. (2014) recently showed that malignancy should be strongly suspected in adrenal masses measuring >20 mm in diameter. In in such cases adrenal glandectomy seem highly recommended (Cook et al. 2014). Similar data were reported also in human studies, where larger lesions (>40 mm) were more likely associated with malignant tumors (Kasperlik-Zeluska et al. 1997). In our study, adrenal lesions <3 mm were usually benign, with 85% (34/40) of the cases histologically diagnosed as cortical hyperplasia. However, small size can still be misleading because other small lesions may be malignant, as seen in the 6 cases of metastases. No data are available on adrenal gland lesions measuring <10 mm because this is the accepted cut-off value of adrenal gland maximum diameter set as an exclusion criterion (Cook et al. 2014).

Among the other ultrasonographic lesion features, abnormal adrenal gland shape may be a useful tool in diagnosis. Nodular shape was significantly associated with cortical hyperplasia, which was highly represented (67/119); however, all the metastatic lesions, the majority of which were cortical carcinoma (14/17), and approximately half of pheochromocytoma, showed a nodular or multinodular ultrasonographic aspects. In contrast, irregular adrenal gland enlargement was seen only in the presence of primary malignant tumors, as previously reported (Besso et al. 1997).

Adrenal laterality (right or left sides) was not found to be a useful predictor for adrenal gland abnormality, whereas bilateral lesions were significantly associated with cortical hyperplasia. As described in previous studies, however, this morphological feature could be confused with malignant metastatic lesions and with rare cases of pheochromocytoma (Cook et al. 2014; Bondyad et al. 1987). According to human and veterinary literature, vascular invasion may be a useful indicator of malignancy. Indeed, all 8 cases (4/17 cortical carcinoma and 4/10 pheochromocytoma) were diagnosed as vascular infiltration of malignant neoplastic cells, but it was not specific for the type of primary adrenal neoplasia (Fan et al. 2014; Besso et al. 1997; Lyon et al. 2002). In our sample, vascular invasion was a specific, but not sensitive ultrasonographic parameter. However, a possible but uncommon association between adrenal hyperplasia and vascular invasion of the distal aorta consequent to the hypercoagulability state often accompanied hyperadrenocorticism has been described (Besso et al. 1997). Consistent with previous findings, adrenocortical carcinomas were generally unilateral, with an equal distribution between sides (Reusch et al. 1991), with atrophy of the contralateral gland in 4 cases, suggesting a hormone-dependent neoplasia, as described elsewhere (Benchekroun et al. 2010). In agreement with previous studies (Besso et al. 1997), cortical adenomas were macroscopically characterized by a nodular pattern. In humans, they usually present as homogeneous hypoechogenic and solid focal or multifocal nodules with well-defined borders (Fan et al. 2014). The only adenoma identified by ultrasound in our study appeared as heterogeneous multifocal nodules with several small areas of inner calcification producing acoustic shadowing. Calcification, necrosis, and hemorrhage are not microscopic features typical of adenoma but they are usually found in large lesions (Besso et al. 1997; Lyon et al. 2002). Differentiation between cortical adenoma and adenocarcinoma based on shape, parenchymal structure, and mineralization was not possible, since 50% of adenoma and carcinoma show calcium parenchymal deposition (Reusch et al. 1991). In half of the cases of pheochromocytoma**,** the tumor was visualized as a large, amorphous encapsulated mass, with irregular margins and loss of normal

shape and parenchymal structure. The remaining lesions presented as solitary or multiple nodules maintaining normal global shape. This pattern of pheochromocytoma has rarely been described (Cook et al. 2014; Besso et al. 1997) and could be confused with other neoplastic lesions. Echogenicity was not specific for malignancy. The mixed echopatterns seemed to correlate with the microscopic evidence of hemorrhagic necrotic areas, as described by Poffenbarger et al. (Poffenbarger et al. 1988). Larger pheochromocytoma seemed to be more heterogeneous, with predominant cystic-necrotic areas. Vascular tumor thrombus was present in 40% of cases, but without distant metastases, consistent with observations by Rosenstein (2000). In humans, pheochromocytoma is usually a benign tumor and as such is not expected to invade adjacent tissues (Fan et al. 2014). In our study, all 10 cases of histologically diagnosed pheochromocytoma were malignant with vascular invasion, confirming its association with malignancy in dogs (Besso et al. 1997; Hoerauf et al. 1999). Adrenal metastasis was frequently bilateral, presenting as multifocal and heterogeneous nodules with irregular margins. In humans, adrenal metastases vary considerably in size and infiltration pattern depending on the type of primary neoplasia from which they originate and are usually described as bilateral lesions (Fan et al. 2014). In our series, the metastatic lesions showed similar characteristics, which could have been due to the high prevalence (85.7%) of the same primary neoplasia. The present study has some limitations: uncommon lesions of canine adrenal glands, such as myelolipomas, which might have led to different conclusions about ultrasound accuracy (Tursi et al. 2005), were not included; furthermore, a high number of dogs that underwent necropsy were affected by the presence of terminal illness, which might bias the population enrolled. In conclusion, as the use of ultrasonography as a diagnostic screening tool continues to increase, we can expect to recognize and diagnose more cases of occult or preclinical adrenal disease. Advances in diagnostic imaging technologies will also improve the identification of small lesions (<10 mm). Although most of them were benign, such as hyperplastic nodules, they could still constitute a challenging clinical issue. Large masses or nodules (>20 mm), associated or

not with vascular invasion, seemed to be a direct parameter to predict malignant adrenal gland neoplasia, while vascular invasion was a specific but not sensitive predictor of malignancy. Lesions <20 mm in diameter and with nodular shape were usually cortical hyperplastic nodules or adenomas. Other criteria, such as adrenal gland laterality, were not specific for predicting malignancy, because only the right side was significantly associated with malignant lesions and bilateral lesions, such as cortical hyperplasia, were more likely to be benign. Clinical signs and laboratory findings of hormonal hypersecretion in the differential diagnosis and workup of adrenal gland lesions require close cooperation between radiologist and endocrinologist, given that only 5 to 15% of adrenocortical tumors are functional and clinical signs of pheochromocytoma are often unspecific (Poffenbarger et al. 1988; Bondyad et al. 1987).

PART 2: BIOCHEMICAL MARKERS FOR ADRENAL GLAND LESIONS

Chapter 6: Inhibin serum and plasma free metanephrines concentrations

INTRODUCTION

Adrenal gland lesions, both neoplastic and hyperplastic, are recognized frequently during abdominal imaging in older dogs, and in many instances are clinically silent (Myers et al. 1997). Imaging of suspected adrenal lesion in dogs is performed with the intention of determining the morphologic features, which may reflect biological behavior, and to look for signs of metastasis. Adrenal lesions may cause clinical signs, depending primarily on their endocrinological activity. Adrenocortical lesions producing cortisol cause signs of canine Cushing's syndrome, including polyuria/polydipsia, polyphagia, hair loss, hepatomegaly, and pendulous abdomen. Medullary lesions, due to epinephrine or norepinephrine-secreting pheochromocytomas, are associated with signs such as weakness, collapse, cardiac dysrhythmia, or hypertension. Endocrinologically inactive adrenal lesions are clinically silent and they have frequently been referred to "adrenal incidentalomas". Algorithms for endocrine testing and imaging procedures are not currently available for investigating the underlying causes of adrenal lesions in dog and an accurate management requires an agreement among results of these diagnostic procedures. Human studies should be demostrated that the presence of an inapparent adrenal lesion does not mean absence of endocrine activity. The patient with an adrenal lesions requires a complete history and physical examination, biochemical evaluation of all pertinent hormones (Mansmann et al. 2004).

The first step in the diagnostic workup of AGL in dog starts with the evaluation of the cortisol secreting activities of adrenal glands, due to the high prevalence of canine hyperadrenocorticism (Reusch et al. 1991; Meyers et al. 1997). A presumptive diagnosis of hyperadrenocorticism in dogs can be made from clinical signs, physical examination, routine laboratory tests, and diagnostic imaging findings, but the diagnosis must be confirmed by use of pituitary-adrenal function tests (Peterson 2007). Screening tests designed to diagnose hyperadrenocorticism include the corticotropin (adrenocorticotropic hormone; ACTH) stimulation test, low-dose dexamethasone

suppression test, and the urinary cortisol:creatinine ratio. More recent, studies described the use of serum inhibin as a biomarker for cortical adrenal gland diseases (Bromel et al. 2013; Sangoi et al. 2010). Inhibin is a glycoprotein synthesized predominantly in ovarian granulosa and testicular Sertoli cells (Findlay 1993; Philips and Woodruff 2004). The primary physiologic roles of inhibin are suppression of follicle-stimulating hormone release from the pituitary gland and regulation of cellular functions in the gonads (Farnworth et al. 1988; Attardi et al. 1989). Circulating inhibin in healthy dogs and humans mainly originates from the gonads (Ishida et al. 1990; Peters et al. 2000). The adrenal glands are known as extragonadal sources of inhibin in humans, but little is known regarding the role of adrenal inhibin (Nishi et al. 1995; Vanttinen et al. 2002). An association of inhibin with adrenocortical disease has been reported by the analysis of serum and adrenal tissue in humans. In vivo and in vitro studies identified both synthesis and secretion of inhibin from adrenocortical tumors in humans, with the highest secretion rates in cortical adenomas associated with Cushing's disease.(Nishi et al. 1995; Nishi et al. 2000). Expression of inhibin in tissue also has been used for differentiating adrenocortical tumors from pheochromocytoma (Sangoi et al. 2010). Immunohistochemical studies in humans identified inhibin alpha expression in adrenocortical hyperplasia, adenomas, and carcinomas, whereas pheochromocytomas were negative (Sangoi et al. 2010; Cho et al. 2001; Zhang et al. 2003). Inhibin concentration in serum has been reported in dogs with adrenal disease, and the value of inhibin as a serum biomarker for adrenal gland tumors has been investigated recently. This study described that adrenocortical tumors and PDH, but not pheochromocytomas are associated with increased serum inhibin concentration (Bromel et al. 2013).

The second step of the diagnostic workup of AGL in dogs should exclude the presence of medullary secreting lesions (Meyers et al. 1997). Medullary hormonal tests should exclude pheochromocytoma in all dogs with adrenal gland lesions, including normotensive patients with apparent clinically silent adrenal masses. The reported incidence of pheochromocytoma is rare in

dogs, but the actual incidence rate is likely higher as the tumor is most often discovered on post mortem examination (Feldman and Nelson 2004; Gilson et al. 1994). The diagnosis of pheochromocytoma is challenging due to the variability of associated clinical signs as the tumor may be hyperfunctional or non-functional and may secrete a wide variety of hormones, predominantly catecholamines (epinephrine, norepinephrine and dopamine). Hypertension most commonly results from a pheochromocytoma, but this condition and other signs may be episodic and difficult to document (Barthez et al. 1997). It has been described that pheochromocytomas contain high concentrations of metanephrines and in many patients with pheochromocytoma, most of the increased urinary and plasmatic levels of catecholamine metabolites result from metabolism directly within tumors, before catecholamines ever reach the circulation (Eisenhofer et al. 2004). In humans, measurement of free (unconjugated) metanephrines in plasma has been shown to be a highly sensitive test for the detection of pheochromocytoma (Lenders et al. 2002; Eisenhofer et al. 2003; Unger et al. 2006). With a diagnostic sensitivity of 98% and specificity of 92%, a negative test result virtually rules out the diagnosis (Eisenhofer et al. 2008). More recently, studies in veterinary medicine demostrated that adequate hormonal testing could identify most canine pheochromocytomas. The diagnosis of pheochromocytoma could be established by the demonstration of elevated 24-h urinary excretion of free catecholamines (norepinephrine and epinephrine) or catecholamine metabolites (normetanephrine and metanephrine) (Kook et al. 2010). The measurement of plasma catecholamines is not recommended, because this method has poor sensitivity and specificity often leading to false-positive results. Meanwhile, plasma-free metanephrines, normetanephrine and metanephrine, have been reported to be more sensitive than other tests, including measurement of catecholamines in 24-h urine for diagnosis of sporadic pheochromocytoma (Hickman et al. 2009). The analytical methods to quantify canine metanephrines plasma concentrations include high pressure liquid chromatography (HPLC) with electrochemical detection, spectrophotometry or fluorometry (Eisenhofer et al. 2004). These

methods can be complex and time-consuming, and therefore may only be available in highly specialised laboratories. In particular, the quantification of canine metanephrine (MN) and normetanephrine (NMN) (the 'metanephrines'), require elaborate methods of extraction and tandem mass spectrometry (LC-MS/MS) and therefore have rarely been determined in canine plasma. Besides these methods, studies in humans described that immunoassays can also be applied to the measurement of plasma-free metanephrines in the diagnosis of catecholamine-secreting tumors (Procopiou et al. 2009).

The aims of this study reported here were to determine the sensitivity and specificity of serum inhibin concentration and plasma-free MN and NMN concentrations in the discrimination of the different canine adrenal gland lesions.

MATERIALS AND METHODS:

The study was composed of dogs attending the Veterinary Teaching Hospital of the University of Turin (Grugliasco, Italy) between January 2014 and October 2016. All dogs selected for inclusion underwent blood biochemical analysis and abdominal ultrasonographic evaluation during their clinical diagnostic workup, followed 24 hours later by a histopathological evaluation of adrenal glands, either post-mortem or after adrenalectomy. For all dogs included in the study a client informed consent was required, according to the requirements outlined by the Animal Care Committee of the Department of Veterinary Sciences of the University of Turin. Patient age, breed, sex and body weight were recorded. Sonographic examinations were performed by the same observer and a real time ultrasonographic machine (My Lab 70 X-Vision, Esaote Biomedica, Firenze, Italy) with electronic multifrequency linear (frequency range 7,5-12 MHz) and micro convex (frequency range 5,5-6,6 MHz) transducers was used. Abdominal ultrasound evaluated measurements, shape and ecogenicity of both adrenal glands and number, dimension and position of adrenal lesions.

All dogs selected for inclusion had an ultrasonographic report describing adrenal gland lesions. For each dog enrolled, history clinical signs, physical examination and the results of haematological, biochemical and urinary analyses were recorded. During blood biochemical analysis, two millilitres of blood from each dog were stored for plasma-free MN and NMN and serum inhibin measurements. Plasma and serum were separated within 10 minutes of collection and stored at -80°C until analyses. Samples were shipped on dry ice to the respective laboratory and thawed immediately before analysis.

Based on the results of the clinical history and routine laboratory exams, hormonal tests were proposed to pet owners with dogs suspected of cortical secreting lesions. Hormonal tests were used to assess cortical adrenal function: urinary cortisol:creatinine ratio (UCCR) combined with low-dose dexamethasone suppression test (LDDST) and/or adrenocortico tropic hormone (ACTH) stimulation test were used. UCCR was performed in all dogs using three consecutive morning urine samples collected by the dog's owner. The cortisol:creatinine ratio was determined in the three urine samples. The mean value of the three UCCR samples was used for diagnosis. The normal reference value for basal UCCR at the laboratory where the samples were analysed is less than 60 x 10–6 ml. A high mean UCCR value was considered consistent with canine hyperadrenocorticism. To increase the diagnostic specificity and to confirm the diagnosis, an ACTH stimulation test or low-dose dexamethasone suppression test (LDDST) was also performed. For ACTH stimulation test, serum cortisol concentrations before and one hour after an intravenous injection of 5 μg/kg tetracosactide (Synacthene; Novartis Pharma) were determined. The ACTH stimulation test result was considered consistent with hyperadrenocorticism when the post-ACTH serum cortisol concentration was more than 19 μg/dL. The diagnosis was also confirmed by a LDDST by collecting blood samples for serum cortisol determination before and four and eight hours after the intravenous injection of 0·01 mg/kg dexamethasone. The failure to suppress cortisol concentration to less than 1.4 μg/dL eight hours after dexamethasone administration was considered consistent

with hyperadrenocorticism of adrenal origin. Dogs with a cortisol concentration less than 1.4 μg/dL or less than 50 per cent of basal cortisol concentration after four hours and a cortisol concentration above 1.4 μg/dL or more than 50 per cent of basal cortisol concentration after eight hours were considered to have PDH because this pattern of suppression is characteristic of the condition. The differentiation between PDH and a functional adrenal tumour was also based on the ultrasonographic appearance of the adrenal glands. The diagnosis of hyperadrenocorticism was made on the basis of specific clinical signs (e.g., polyuria, polydipsia, alopecia, pendulous abdomen, and weakness), results of complete blood count, serum biochemical analysis, and urinalysis (e.g., leukocytosis, lymphopenia, eosinopenia, high alkaline phosphatase activity, and dilute urine specific gravity) and abnormal results of at least 2 of 3 endocrine screening tests (ACTH stimulation test,a low-dose dexamethasone suppression test, or urine cortisola-to-creatinine ratio determination).

Only dogs with histopathological evalutation of both adrenal glands, within 24 hours of sonographic examination, were included. The adrenal glands were removed and placed in 10% buffered formalin. After 7 days the formalin fixed glands underwent histopathological examinations. From histological results, all dogs enrolled were divided in three groups: dogs with nonadrenal lesions (NAL), dogs with adrenocortical lesions (ACL) and dogs with pheochromocytomas (PHEO). Healthy dogs were used as controls and were privately owned. All control dogs were considered healthy on the basis of lack of clinical signs of adrenal disease and unremarkable findings of physical examination.

Among the blood samples stored at -80°C, all dogs were evaluated for the plasma-free MN and NMN measurement and only the neutered dogs were selected for the serum inhibin evaluation.

Inhibin concentration was measured in serum by Inhibin Alpha-Subunit (1–32) (Porcine) EIA kit® (Phoenix Pharmaceuticals, Inc., Karlsruhe, Germany). Analysis was performed as recommended by the manufacturers' protocols. Inhibin levels were measured by interpolation of the standard curve of

log standard concentrations versus normalised absorbances. All the samples were analyzed in duplicate. Serum samples were appropriately diluted with kit buffers for inhibin assays to fall within the concentration range of the standard curve. The sensitivity of the inhibin assays was 45.4 pg ml−1.

Plasma-free MN and NMN were measured by liquid chromatography tandem mass spectrometry (LC-MS - Acquity Waters UPLC system, Manchester, UK) at the Baldi and Riberi Laboratory for Clinical Biochemistry at the University Hospital of Molinette (Turin, Italy). Solid phase extraction was performed using Oasis WCX μElution 96-well plates, preconditioned with 200 μL of HPLC grade methanol and 200 μL of 10 mmol/L ammonium phosphate (pH 6.5). 100 μL of calibrators, quality controls and patients' samples were thoroughly mixed with 100 μL of 10 mmol/L ammonium phosphate buffer and 25 μL of working internal standards. After application of calibrators, quality controls and patient's samples, the wells were sequentially washed with 200 μL of HPLC grade water, 200 μL of methanol and 200 μL of HPLC grade acetonitrile containing 0.2% formic acid. The metanephrines and their stable isotope internal standards were then eluted with 3Å~25 μL volumes of freshly prepared 2% formic acid in 95:5 acetonitrile:water, collecting the eluate directly into the sample collection plate. Plates were sealed with heat-sealing film and placed in the autosampler chamber set at 14 °C. Using the Oasis WCX μElution 96-well plates flow rates for the load and elute steps should not exceed 1.0 ml/min whilst for all other steps, flow rates up to 5 mL/ min can be tolerated. The eluate collected from the samples was subject to analysis by LC–MS/MS using aWaters Acquity UPLC/Quattro Premiermass spectrometerwith an injection volume of 35 μL. Data acquisition and control of the MS/MS system were performed using MassLynx™v4.0 software with automated data processing by the QuanLynx Application Manager. Chromatographic analysis was performed using a binary gradient on an Atlantis HILIC Silica (3 μm) 2.1Å~30mm column (Waters) at 25 °C with mobile phases of 100 mmol/L ammonium formate in water adjusted to pH 3.0 with formic acid (eluent A) and acetonitrile (eluent B). Gradient applied

to the column was: 0 min 5% A/95% B; 1.0 min 30% A/70% B; 2.0 min 30% A/70% B; 2.1 min 5% A/95% B. The flow rate was 0.35 mL/min, with normetanephrine and metanephrine and their deuterated analogues eluting at 2.0 min, with a total run time of 3.5 min.

Plasma-free MN and NMN were also determined using an EIA commercial kit manufactured by IBL International GmbH, Hamburg, Germany (MetCombi Plasma ELISA Metanephrine/Normetanephrine 2 x 96 Best.). Analysis was performed as recommended by the manufacturers' protocols. Plasma-free MN and NMN levels were measured by interpolation of the standard curve of log standard concentrations versus normalised absorbances. All the samples were analyzed in duplicate. Plasma samples were appropriately diluted with kit buffers for inhibin assays to fall within the concentration range of the standard curve.

Statistical analysis was performed by R software (version 3.1.2, R Core Team 2015). The Shapiro-Wilk normality test was used to assess normality of the data. For comparison of plasma-free MN and NMN and serum inhibin concentrations among the 4 groups of dogs (NAL, ACL, PHEO and healthy group), the Kruskal- Wallis ANOVA was used, and if differences were significant ($P < 0.05$), that analysis was followed by posthoc Mann-Whitney tests. Correlations between LC-MS and EIA results were evaluated by the use of Spearman's rank correlation coefficient. For all statistical analyses, a P value of 0.05 was considered significant and all results were reported as median and range. The sensitivity and specificity of plasma-free MN and NMN and serum inhibin concentrations in the diagnosis of pheochromocytoma were calculated. Plasma-free MN and NMN and serum ihibin results were combined for all dogs without pheochromocytoma (healthy, NAL and ACL groups) to create the reference group. The 2.5th and 97.5th percentiles were calculated for both concentrations within this group by nonparametric methods to give ranges incorporating 95% of measured values. The 97.5th percentile for fMN and fNMN concentration in this group was used as a cut-off point to determine the sensitivity and specificity of fMN and fNMN concentrations for the diagnosis of pheochromocytoma. Boxes were generated by SigmaPlot (Systat Software Inc, San

Jose, CA) and represent the interquartile ranges from the 25th to the 75th percentiles. Horizontal lines within the boxes indicate median values. Whiskers represent the 10th and 90th percentiles.

RESULTS:

Fifty eight dogs were enrolled: 24 dogs with adrenocortical lesions (ACL), 15 dogs with nonadrenal lesions (NAL), 12 healthy dogs and 7 dogs with pheochromocytoma (PHEO).

Dogs with adrenocortical lesions (ACL)

Twenty four dogs with cortical lesions confirmed by histology were included; 6 dogs were intact male and 18 were female (14 spayed). Age ranged between 7.0 and 17.0 years (median 13.0) and body weight between 3.5 and 50.0 kg (median 10.0). Breeds included Yorkshire Terrier (3), West Highland White Terriers (2), Shih Tzu (2), German Shepherd (1), German Hound (1), Flat-Coated Retriever (1), American Straffordshire Terrier (1), Pomeranian (1), Italian Mastiff (1) and 11 mixed-breed dogs. Twelve dogs showed clinical signs consistent with hyperadrenococrticism including polyuria, polydipsia, polyphagia, skin problems, weakness, abdominal enlargement. All dogs were tested for the hormonal specific tests to assess cortical adrenal function: urinary cortisol:creatinine ratio (UCCR) combined with low-dose dexamethasone suppression test (LDDST) and adrenocortico tropic hormone (ACTH) stimulation test. Urinary cortisol:creatinine ratio showed in all cases positive resulsts. LDDS and ACTH stimulation test yielded a positive results in only 10 dogs (5 dogs described as ATS and 5 as PDH). Dogs affected by PDH showed bilateral symmetrical appearance of the adrenal glands determined by ultrasonography. Histopathology of dogs affected by PDH showed in all cases bilateral multinodular cortical hyperplasias. Hyperadrenocorticism caused by an adrenocortical tumor (AT) was identify in 5 dogs on the hormonal specific tests and the finding of an adrenal lesions by ultrasonography. Ultrasonographic maximum diameter of adrenal lesions ranged between 6.0 mm and 46.0 mm, with a median of 24 mm. Functional adrenocortical tumor was confirmed by histologic examination of the adrenal glands after unilateral right adrenalectomy in all 5 dogs and histopathologic exam

described an cortical carcinoma. Among dogs without clinical signs of hyperadrenocorticism, histological examination showed 8 unilateral adrenocortical carcinomas (4 right and 4 left adrenal glands), 2 cortical nodular hyperplasias, 1 adenoma and 1 purulent adrenalitis.

Dog with Pheochromocytoma (PHEO)

Seven dogs with PC were included; 4 of them were male (2 castrated) and 3 were females (2 spayed). Age ranged between 6.0 and 12.5 years (median 11) and body weight between 8.7 and 44.0 kg (median 17.5). Breeds included Beagle (1), English Pointer (1), Dachshund (1), and 4 mixed-breed dogs. In all dogs enrolled the diagnosis was confirmed based on histopathologic examination of the adrenal gland by a complete necropsy. On the clinical reports, 5 dogs showed various clinical signs indicative of pheochromocytoma, including weakness, abdominal panting, tachycardia, tachypnea, weight loss, polyuria/polydipsia and anorexia. A dorsocranial abdominal palpable mass was the only physical examination finding in the 2 dogs without clinical signs. Hemogram abnormalities include anemia, leukocytosis and thrombocytosis. Five dogs showed stress leukogram characterized by neutrophilia, lymphopenia and monocytosis. Serum biochemistry panel results were always unremarkable. Two dogs had been presented with clinical signs of suspected hyperadrenocorticism and had a negative low-dose dexamethasone suppression test (LDDST) and ACTH stimulation test. By means of ultrasonography unilateral adrenal enlargement was identified in 3 dogs (left adrenal gland in all dogs), in 4 dogs bilateral adrenal enlargement was found. Maximum diameter of adrenal lesions ranged between 37.0 mm and 72.0 mm (median 42.0 mm). Two dogs showed bilateral adrenal enlargement with local invasion of caudal vena cava with *tumor thrombus* phenomena were described. No distant metastases of pheochromocytoma were found. Four dogs showed the concomitant presence of other malignant neoplasmas, including splenic and hepatic hemangiosarcoma, multicentric lymphoma and renal carcinoma.

Dogs with nonadrenal lesions (NAL)

Fixteen dogs with NAL were included: 11 dogs were male (4 castrated) and 4 dogs were intact female. Age ranged between 1.0 and 14.0 years (median 9.2) and body weight between 4.1 and 45 kg (median 23.8). Breeds enclosed Flat Coated Retriever (1), Beagle (1), Golden Retrivers (1), Pomenarian (1), West Highland White Terrier (1), Rottweiller (1), Dachshund (1), Standard Poodle (1), Shetland (1), German Shepherd (1), Border Collie (1), Greyhound (1), Italian Hound (1), and 2 mixed-breed dogs. The dogs were suffering from cardiac failure (3), lung carcinoma (2), neurologic diseases (2), sertolioma (1), insulinoma (1), gastrointestinal foreign body (1), chronic kidney disease (1), hemangiosarcoma (1), hepatic shunt (1), multicentric lymphoma (1) and immune-mediated anemia (1). In 14 dogs both adrenal glands were either found to be normal on ultrasonography. Only in one dog, ultrasonography showed nodular lesions (maximum diameter 10 mm) on cranial and middle portion of both adrenal glands, described as metastatic lesions by histology.

Healthy Dogs

Twelve healthy client-owned dogs including 5 males (2 castrated) and 7 females (3 spayed) with a median body weight of 24.0 kg (range, 8.0– 57.0 kg) and a median age of 3.0 years (range, 1.0 – 6.0 years) were used. Breeds included Belgian Malinois (2), Golden Retriever (1), Australian Shepherd (1), English Mastiff (1), Dalmatian (1), and 6 mixed-breed dogs. They were determined to be healthy on the basis of history and results of physical examination, CBC, serum biochemical profile, and abdominal ultrasonography. None of the dogs had received any medication for at least 8 weeks before inclusion in the study except routine vaccination, deworming and heartworm prophylaxis.

Results of serum inhibin and plasma-free MN and NMN concentrations for dogs with NAL, ACL, PHEO, and healthy dogs were summmarized in **Tables 8-9.**

There was a marked overlap in plasma-free metanephrine values relived by EIA and LC-MS method among all groups (**Figure 22**). Meanwhile, plasma-free normetanephrine values were

significantly higher in dogs with pheochromocytoma compared to dogs with ACL, NAL and healthy dogs (**Figure 23**). Statistical significant differences between plasma-free normetanephrine (NMN) evaluated by EIA and LC-MS methods were summarized in **Table 10**.

The comparison of plasma-free NMN values between EIA and LC-MS evaluations showed a significant positive correlation ($r = 0.86$, $P < 0.001$) as well as for plasma-free MN values ($r = 0.63$, $P < 0.001$).

Serum inhibin concentration, using a cut off of 9.31 ng/mL, retained a 100% of sensivity and 79% of specificity (AUC=0.89) for the diagnosis of pheochromocytoma.

Plasma-free normetanephrine using a cut off of 2549 pmol/L, evaluated by LC-MS and of 5155 pmol/L evaluated by EIA retained a 85.7% of sensivity and 63% of specificity (AUC=0.86) for the diagnosis of pheochromocytoma.

Table 8: Results of serum inhibin concentrations in neutered dogs with pheochromocytoma (PHEO), with adrenocortical lesions (ACL), with nonadrenal lesions (NAL), and in healthy dogs (HEALTHY).

Median (Range) **(ng/mL)**	PHEO (n=3)	ACL (n=10)	NAL (n=3)	HEALTHY (n=6)
INHIBIN	5.08 * (0.50–7.83)	23.66 (5.70– 75.16)	32.65 (15.20-57.50)	13.10 (2.84–58.84)

*significant statistical difference comparing PHEO vs ACL group (P=0.02).

Table 9: Median and ranges values of plasma-free metanephrine (MN) and normetanephrine (NMN) evaluated by EIA and LC-MS methods in dogs with pheochromocytoma (PHEO), with adrenocortical lesions (ACL), with nonadrenal lesions (NAL), and in healthy dogs (HEALTHY).

	PHEO (n=7)	**ACL** (n=24)	**NAL** (n=15)	**HEALTHY** (n=20)
Plasma-free NMN-EIA (pmol/L)	17425 (1556-58643)*	4401 (1202–9352)	7249 (1483-11646)	2735 (1066-3737)
Plasma-free NMN-LC-MS (pmol/L)	32744 (1584-73369)*	2141 (459-8291)	3337 (1153-8756)	1081 (429-2476)
Plasma-free MN-LC-MS (pmol/L)	4935 (315-14990)	480 (194-2574)	1118 (226-2700)	962 (309-1971)
Plasma-free MN-EIA (pmol/L)	5368 (391-14997)	362 (175-4246)	1101 (129-2679)	646 (169-4656)

* statistically significant differences ($P < 0.05$) between all groups.

Table 10: Statistical significant differences (p-value<0.05) between plasma-free normetanephrine (NMN) evaluated by EIA and LC-MS methods in dogs with pheochromocytoma (PHEO), with adrenocortical lesions (ACL), with nonadrenal lesions (NAL), and in healthy dogs (HEALTHY).

P-Values		**ACL** (n=24)	**NAL** (n=15)	**HEALTHY** (n=20)
Plasma-free NMN - EIA	**PHEO** (n=7)	0.0041	0.0071	0.0039
Plasma-free NMN -		0.0001	0.0003	0.0006

LC-MS				

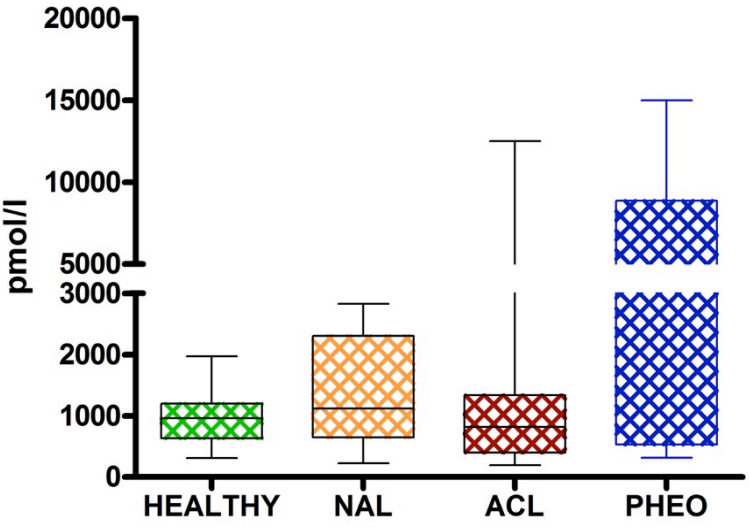

Figure 22 Box plot of plasma-free metanephrine (MN) evaluated by LC-MS method in dogs with pheochromocytoma (PHEO, n=7), with adrenocortical lesions (ACL, n=24), with nonadrenal lesions (NAL, n=15), and in healthy dogs (HEALTHY, n=20). Data are given as the means (box indicates ± SD) and range (indicated by the bar).

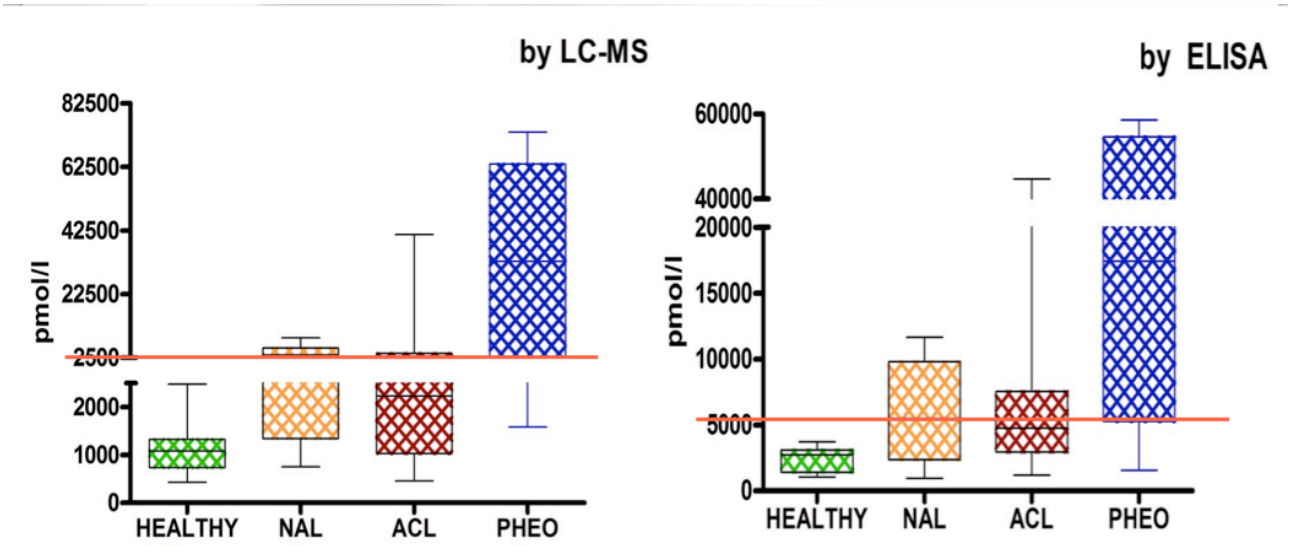

Figure 23: Box plots of plasma-free normetanephrine (NMN) evaluated by EIA and LC-MS methods in dogs with pheochromocytoma (PHEO, n=7), with adrenocortical lesions (ACL, n=24), with nonadrenal lesions (NAL, n=15), and in healthy dogs (HEALTHY, n=20). Data are given as the means (box indicates ± SD) and range (indicated by the bar). Red line reports the cut off of 2549 pmol/L (by LC-MS) and of 5155 pmol/L (by EIA)

DISCUSSION:

Dogs with adrenal gland lesions, identified during a diagnostic imaging modality, requires a complete history, physical examination and biochemical evaluation of all pertinent adrenal secreting hormones (Mansmann et al. 2004). Due to the higher prevalence of primary adrenal cortical lesions, the first step in the diagnostic workup of AGL in dogs should start with the evaluation of the cortisol secreting activities of adrenal glands. A presumptive diagnosis of hyperadrenocorticism in dogs can be made from clinical signs, physical examination, routine laboratory tests, and diagnostic imaging findings, but the diagnosis must be confirmed by use of pituitary-adrenal function tests (Peterson 2007). The most commonly used screening tests for hyperadrenocorticism in dogs are the urine cortiso1:creatinine ratio, the low-dose dexamethasone suppression test and ACTH stimulation test. Unfortunately, none of these diagnostic tests used in dogs with suspected hyperadrenocorticism were totally reliable, and both false-positive and false-negative results were common (Peterson 2007). The use of serum inhibin concentration and plasma-free metanephrine (MN) and normetanephrine (NMN) as biomarkers for distinguish adrenal gland lesion origins could represent a new useful method to help the veterinarian challenged when attempting to properly interpret the dog's hormonal tests results. Determination of these biomarkers requires a single blood sample, no special sample handling, and no injections for additional pituitary-adrenal function testing (Bromel et al. 2013).

In our study, both serum inhibin concentration and plasma-free normetanephrines were able to distinguish adrenal medullary from adrenal cortical lesions.

In neutered dogs affected by adrenal medullary lesions (PHEO), serum inhibin was significant lower comparing to dogs affected by adrenal cortical lesions (ACL) ($p=0.02$). Dogs with adreno-cortical lesions (ACL) had median serum inhibin concentration >4.5 times higher than neutered dogs with pheochromocytoma, suggesting that adrenocortical tumors synthesize and secrete inhibin into the circulation despite the adrenal medullary tumors. Serum inhibin concentrations in dogs with

adrenal cortical lesion (n=24) did not distinguish dogs with PDH from dogs with adrenocortical tumors, probably due to the small sample size included with a diagnosis of hyperadrenocorticism based on hormonal specific tests (5 dogs with ATS and 5 dogs with PDH).

Our results were similar to those described in previous study, where in neutered dogs with adrenal-cortical lesions, median inhibin concentration was significantly higher (>7.5 times more) than in dogs with PHEO and healthy dogs (Bromel et al. 2013). At the same time, as in the earlier study, inhibin concentration in the 3 neutered dogs included and affected by pheochromocytoma were almost undetectable and did not differ from serum inhibin concentration in healthy dogs (Bromel et al. 2013). The small number of neutered dogs with PHEO was a limitation of the previous study (n=3) as well as in this study.

Our results showed an addiction data regarding the overlap between the dogs without adrenal lesions (NAL) and dogs with adrenal cortical lesions (ACL), describing that serum inhibin could be use as a marker for the diagnosis of pheochromocytoma. Using a cut off of of 9.31 ng/mL, inhibin concentration showed a 100% of sensivity and 79% of specificity (AUC=0.89), similar to that reported by a previous study in dogs (sensibility 100% and specificity 88.9%) (Bromel et al. 2013). This means that diagnostic sensitivity would decrease if the cut-off were changed.

In the only other study on the serum ihibin, as adrenal disease biomarker in dogs, published prior to this report, the inhibin concentration was measured by radioimmunoassay (RIA) using an antibody raised against 31 kDa bovine inhibin. The inhibin concentrations of our sample were 10-100 times higher that those reported in the previus study, probably due to higher diagnostic accuracy of EIA kit used compared to RIA methods.

Unfortunately, inhibin concentration is not applicable in sexually intact dogs because gonadal inhibin in serum cannot be differentiated from inhibin secreted by adrenocortical tumors (Bromel et al. 2013). In addition, circulating inhibin concentrations are high in dogs with testicular Leydig cell

or Sertoli cell tumors, that have to be take into consideration in the diagnostic workup of the adrenal gland lesions (Peters et al. 2000; Grootenhuis et al. 1990; Taniyama et al. 2001).

In summary, inhibin concentration in serum appears to be a future valuable biomarker to distinguish adreno-cortical from adrenal-medullary lesions in neutered dogs. If serum inhibin is most like to be an undetectable value, adrenal lesions are more like to be pheochromocytoma, especially if associated with higher level of plasma-free normetanephrine and appropriate clinical signs.

Further studies are needed to increase the number of population included, indeed reference intervals or cut off values of inhibin concentration should best be established in a larger population with an wider age range for each group.

In this study, we also investigated the diagnostic accuracy of biomarkers as plasma-free metanephrines in differentiating dogs with adrenal cortical and adrenal medullary lesions. In veterinary medicine, limited biochemical tests are available to the clinicians for the diagnosis of canine pheochromocytoma. Currently, the diagnosis is based on plasma or urinary measurement of the direct secretory products of the adrenomedullary parenchima or their metabolites, specifically catecholamines or their metanephrine derivatives (Salesov et al. 2015; Gostelow et al. 2013).

This study confirmed that the plasma-free NMN concentrations is a sensitive screening marker for the detection of pheochromocytoma in dogs as previously described (Salesov et al. 2015; Gostelow et al. 2013). Plasma-free NMN concentrations was a suitable biomarker despite the plasma-free MN in distinguishing dogs with pheochromocytoma from healthy dogs, dogs with cortical lesions and dogs with nonadrenal lesions. Plasma-free NMN concentration was consistently higher in the dog with pheochromocytoma than in the other groups. Plasma-free NMN concentration in pheochromocytoma showed no overlap with values measured in other groups than was shown for plasma-free MN concentration**.** In this study, we also investigated the diagnostic accuracy of a new EIA for plasma-free MN and NMN in comparison with the current analytical technique (LC-MS). In our study, the EIA technique has been tested against metanephrines measurements by liquid

chromatography coupled with tandem mass spectroscopy (LC-MS), because this last technique for plasma metanephrines is not routinely available in veterinary practices. Canine plasma-free MN and fNMN concentrations are much higher than those encountered in human patients, making it able to be dected with less accurate technique as EIA methods. In our study, plasma fNMN measurement by EIA appears to be an accurate method of identifying dogs with pheochromocytoma as well as by LCMS/MS. Further studies should be performed to provide dog-specific reference ranges by EIA technique.

Chapter 7: Summarizing discussion and conclusions

Our study discovered a high prevalence of adrenal gland lesions in dogs (27%), described by routinely abdominal ultrasound. The majority of them (about 60%) were displayed as nodular or solid mass, which has been commonly associated in literature with neoplastic features. This data could be due to the concurrent high prevalence of dogs affected by oncological and endocrinological disease (31.5% and 16%, respectively) presenting in our population, generating a bias on our results. Previous study described a prevalence of 4% of adrenal gland lesions (AGL) discovered incidentally during abdominal ultrasound (Cook et al. 2014) and more than 75% of these cases were histologically proven to be metastatic. Therefore, it is possible that some or many dogs of the AGL group, with concurrent neoplasia, had metastatic lesions affecting adrenal glands. But only the histological evaluations, developed in the second phase of our studies, give us the actual confirmation. Based on histological findings, the prevalence of adrenal gland lesions were confirmed to be high, reporting to be 18.1%, of which 81.7% were primary adrenal neoplasia and 18.3% metastatic lesions. The most frequent neoplastic type was adrenocortical carcinoma (44.7%), followed by pheochromocytoma (26.3%), metastases (18.4%), and adrenocortical adenoma (10.5%). A high prevalence (32%) of adrenal cortical hyperplasia was also noted. In the second phase of our study, we were also able to compare ultrasonographic and macroscopic evaluation of the adrenal glands, showing high specificity (100%) but low sensitivity (63.7%) for identifying the adrenal lesions, which improved with increasing lesion size. The high specificity of the ultrasonographic evaluation of adrenal lesion was due to the fact that no one of the healthy glands was described as pathological and the low sensitivity was due to the fact that 6 out of 17 cases of cortical adenocarcinoma, 4 out of 7 metastasis, 1 out of 10 as pheochromocytoma, were not correctly displayed. In the opposite site, nearly all lesions (20/22) >10 mm were correctly detected by ultrasound. Ultrasonographic abnormal appearance of size, shape, laterality, and echotexture of adrenal glands may aid in diagnosis, but these features alone were not pathognomic. Lesion size

was the most direct ultrasound predictive criterion. Large and irregular masses seemed to be better predictors of malignant neoplasia and lesions <20 mm in diameter and nodular in shape were often identified as cortical hyperplastic nodules or adenomas. Based on the discovered importance of adrenal lesion size, our studies investigated also the agreement between ultrasonographic and gross adrenal glands measurements. Our results reported that the thickness of the caudal pole of both glands is a much more reliable parameter to distinguish between normal and pathological glands, according with the less intra- and inter-observer variability previous described (Barberet et al., 2010; de Chalus et al., 2013; Soulsby et al., 2014). For these reasons, the caudal pole appears to be the most reliable and recommended measurement for clinical evaluation, when taking into account the reference range of size for each canine body weight category, previously described (Bento et al. 2016).

In support of ultrasonographic modality, dogs with adrenal gland lesions correctly displayed, requires a complete history, physical examination and biochemical evaluation of all pertinent adrenal secreting hormones. Currently, none of these diagnostic exams have been described to be totally reliable, and both false-positive and false-negative results were common (Peterson 2007). In order to increase the accuracy of the diagnostic workload of the adrenal gland lesions in dogs, our studies have deepened the use of serum inhibin concentration and plasma-free metanephrine (MN) and normetanephrine (NMN) as biomarkers for distinguish adrenal gland lesion origins. Our results showed as both serum inhibin concentration and plasma-free normetanephrines were able to distinguish correctly adrenal medullary from adrenal cortical lesions. Plasma-free NMN concentrations in dogs with pheochromocytoma were significatly higher than in dogs with cortical lesions, nonadrenal lesions and healthy dogs. On the other hand, undetectable or low level of inhibin in serum in dogs with adrenal gland lesions was highly supportive of the presence of adrenal pheochromocytoma. In conclusion, these biomarkers could represent a suitable method to help the veterinarian when adrenal gland lesions were displayed by diagnostic imaging methods and the

properly interpretation of hormone secreting tests were challenged. Although our studies have examined ultrasonographic and biochemical features in an attempt to define simple criteria to distinguish the adrenal lesion origins, unequivocal strategies in differential diagnosis are still lacking. Moreover, the frequency of these adrenal lesions is increasing and the cost incurrent for the diagnosis in dogs are becoming an important aspect. Nevertheless, the synergic interpretation of the clinical, laboratory and diagnostic imaging results remains mandatory to obtain a better characterization of the adrenal gland lesions.

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