Received: 15 March 2021 | Accepted: 16 November 2021

DOI: 10.1111/jvim.16320

CASE REPORT

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Cushing's syndrome caused by intra-adrenocortical adrenocorticotropic hormone in a dog

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Funding information
University of Buenos Aires-UBACyT, Grant/Award Number: 20720170100006BA

Abstract

A 13-year-old Labrador retriever was diagnosed with Cushing's syndrome (CS) caused by primary bilateral nodular adrenocortical hyperplasia with adrenocorticotropic hormone (ACTH) expression. The pituitary origin of CS was ruled out by suppression of plasma ACTH concentration and absence of a proliferative lesion on histological evaluation of the pituitary gland using periodic acid-Schiff (PAS) staining, reticulin staining, and immunostaining for ACTH. A pheochromocytoma also was found at necropsy examination. On histological evaluation of both adrenal glands, at the junction of the fascicular and glomerular zones, multiple cell clusters distributed in both hyperplastic adrenal cortices expressed ACTH, whereas the pheochromocytoma cells did not. These results indicate that a disease similar to primary bilateral macronodular adrenocortical hyperplasia in humans also occurs in dogs, with intra-adrenocortical expression of ACTH, glucocorticoids excess, and clinical signs of CS. Therefore, the term ACTH-independent could be inappropriate in some cases of bilateral adrenocortical hyperplasia and suppressed plasma ACTH concentration in dogs.

KEYWORDS
ACTH-independent, adrenal hyperplasia, adrenocorticotropic hormone, ectopic ACTH, hypercortisolism, pheochromocytoma

1 | INTRODUCTION

According to its origin, Cushing's syndrome (CS) in dogs generally is classified as pituitary (ACTH-dependent) or adrenal (ACTH-independent).[1] An additional source of pituitary ACTH (ectopic ACTH secretion syndrome) originating from a neuroendocrine neoplasm is also a reported cause of ACTH-dependent CS in dogs.[2-5] Typically, adrenal CS results from a cortisol-secreting adrenocortical tumor (adenoma or carcinoma),[6] and, exceptionally, from expression of aberrant adrenal receptors such as the glucose-dependent insulinotropic peptide (GIP) receptor which is involved in food-dependent hypercortisolism.[7]

Primary bilateral adrenocortical hyperplasia (macronodular and micronodular) occurs in approximately 10% of cases of ACTH-independent CS in humans.[8] Primary bilateral macronodular adrenocortical hyperplasia (PBMAH) may present as an isolated disorder or as part of multiple endocrine neoplasia syndromes. The pathophysiological events that account for adrenal hyperplasia and hyperfunction include germline mutations affecting diverse genes such as *MEN1*, *GNAS1* and mainly *ARMC5*, expression of aberrant G protein-coupled receptor and paracrine action of intra-adrenocortical ACTH production.[9,10] At present, few cases of CS caused by intra-adrenal ACTH

Abbreviations: 3βHSD, 3beta-hydroxysteroid dehydrogenase; ACTH, adrenocorticotropic hormone; CS, Cushing's syndrome; GIP, glucose-dependent insulinotropic peptide; LDDST, low-dose dexamethasone suppression test; PBMAH, primary bilateral macronodular adrenocortical hyperplasia; UCCR, urinary cortisol/creatinine ratio; VMA/Cr, urinary vanillylmandelic acid/creatinine ratio.

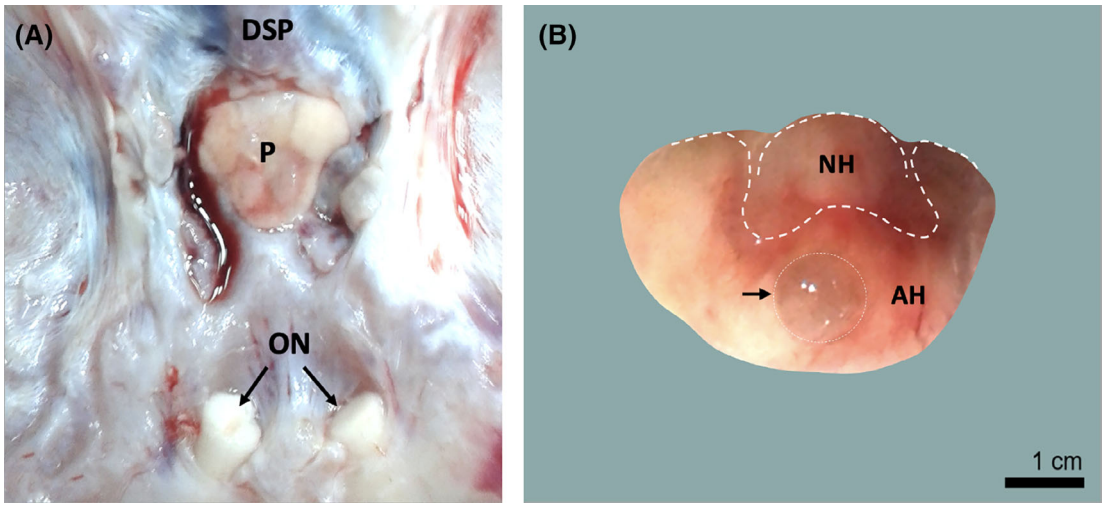

FIGURE 1 Macroscopic postmortem appearance of the base of the brain showing the hypophysis gland (P) in normal position and below the dorso-sellar process (DSP) of the spheroid bone (A). Macroscopic appearance of the cranial surface of the pituitary gland, showing the neurohypophysis (NH) and a small cyst in the adenohypophysis (AH) (B). Optic nerves (ON)

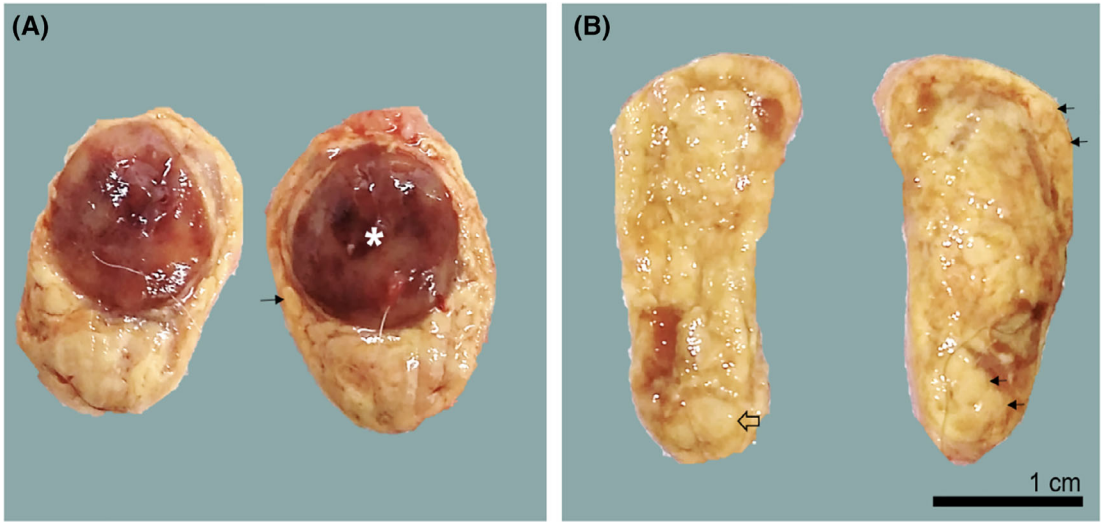

FIGURE 2 Macroscopic appearance of both adrenal glands with multiple small yellowish nodules (thin arrows, 1-2 mm) and some larger nodules (empty arrow, 4 mm) in the sagittal section. Left (A) and right (B) adrenal gland, respectively. The left adrenal gland shows a well-defined round reddish tumor (asterisk) of 11 mm in diameter

production by steroidogenic cells in PBMAH have been reported in humans.[9-12]

In dogs, to date, primary bilateral adrenocortical hyperplasia (ACTH-independent) is not considered as a cause of CS.[6] To our knowledge, the dog reported here represents the first case of primary bilateral nodular adrenocortical hyperplasia associated with intra-adrenocortical ACTH and CS in a dog.

2 | CASE DESCRIPTION

A 13-year-old neutered male Labrador retriever (body weight, 29 kg) was referred for evaluation clinical signs compatible with CS. The owner reported at least a 6-month history of polyuria, polydipsia, polyphagia, muscular weakness, weight loss, panting and sporadic but recurrent episodes of agitation and nervousness. A mild pendulous abdomen and atrophy of the temporal muscles and proximal muscles of all 4 limbs were the only relevant findings in physical examination. Abnormal laboratory findings included increases in the plasma activity of alkaline phosphatase (1693 U/L; reference interval [RI], <250 U/L) and alanine aminotransferase (143 U/L; RI, <50 U/L) and increased total cholesterol concentration after 10 hours of fasting (255 mg/dL; RI, <220 mg/dL). A low urine specific gravity (USG, 1.018) with increased urinary protein/creatinine ratio (UPC, 0.6; RI, <0.2) and increased systolic blood pressure (185 mm Hg; RI, <160 mm Hg) also were present. On abdominal ultrasound examination, both adrenal

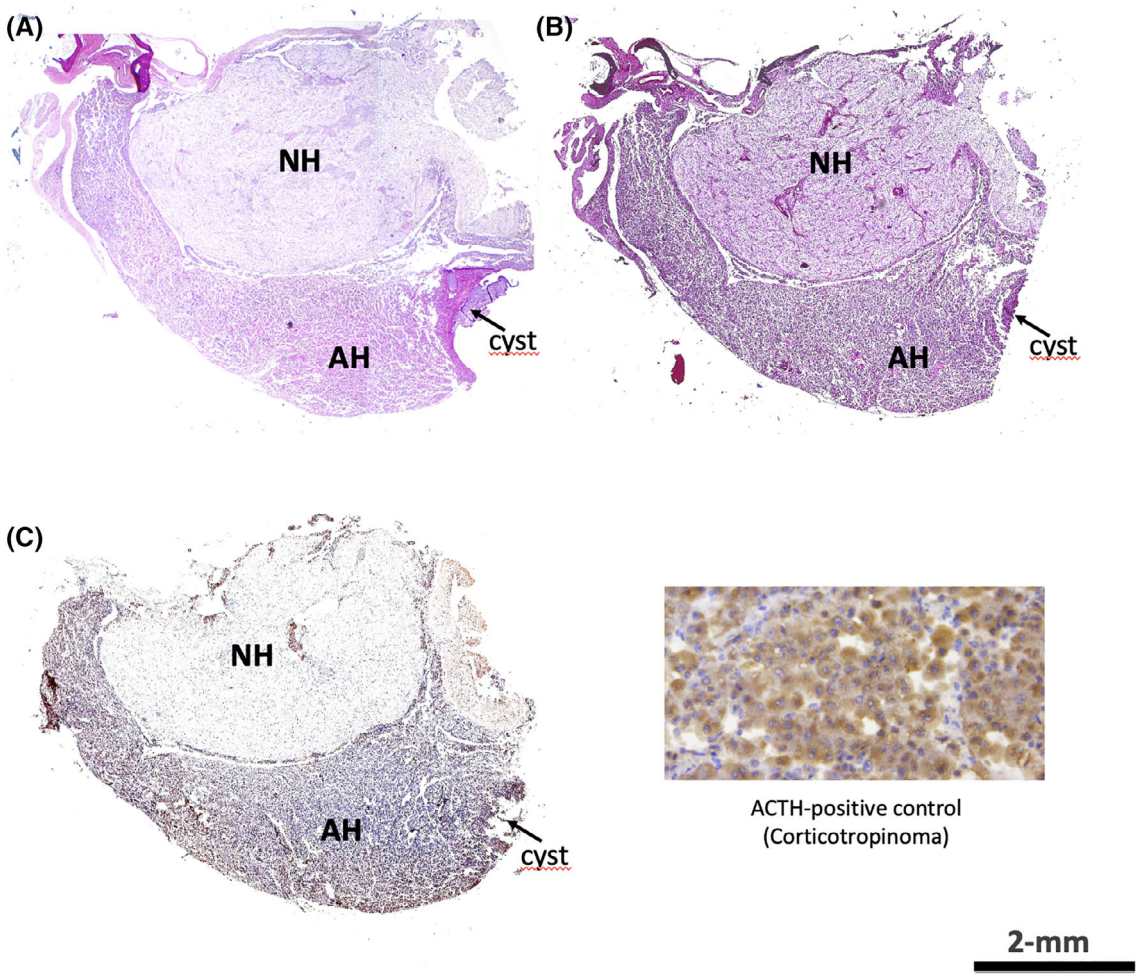

FIGURE 3 Microscopic images of sagittal sections of the pituitary gland without evidence of focal proliferative lesion in hematoxylin-eosin (A), periodic acid-Schiff (PAS) (B), and immunohistochemical staining for adrenocorticotropic hormone (ACTH) (C). In all stains, the presence of the pituitary cyst in the cranial position of the adenohypophysis (AH) was evident. NH, neurohypophysis

glands were enlarged (>10 mm in caudal pole in the sagittal plane; RI, ≤6.8 mm),[13] and a well-circumscribed nodule 12 mm in diameter was detected in the left adrenal gland. Based on the aforementioned findings, CS was suspected, and measurement of the urinary cortisol/creatinine ratio (UCCR) and low-dose dexamethasone suppression test (LDDST) were performed. The UCCR was increased (84×10^{-6}; RI, $<15 \times 10^{-6}$) and plasma cortisol concentration post-LDDST was not suppressed (baseline, 3.4 μg/dL; RI, 0.6-6.0 μg/dL; 4 hour, 2.2 μg/dL; RI, <1.4 μg/dL; 8 hour, 1.9 μg/dL; RI, <1.4 μg/dL). Results of the UCCR and LDDST were consistent with a diagnosis of CS. The finding of a nodule in the left adrenal gland on ultrasound examination raised suspicion of a cortisol-secreting adrenal tumor. However, because of the enlargement of both adrenal glands, pituitary magnetic resonance imaging (MRI) and plasma endogenous ACTH concentration were performed to rule out a concurrent pituitary tumor (corticotropinoma). Plasma ACTH was suppressed (<10 pg/mL; RI, 10-65 pg/mL) and MRI was declined by the owner. Therefore, the diagnosis of ACTH-independent CS caused by a left adrenal tumor was considered and unilateral adrenalectomy was proposed. Surgery also was declined, but treatment with 1 mg/kg PO q12h of trilostane (Oncovet TL, Chemovet Lab., Argentina), a 3β-hydroxysteroid dehydrogenase (3βHSD) competitive inhibitor, was initiated.

After 4 weeks of treatment, the owner reported a decrease in water intake and urine output, but signs of agitation and nervousness were becoming more frequent. In addition, systolic blood pressure (175 mm Hg) and UPC (0.45) remained high although trilostane treatment caused remission of the clinical signs of CS. Although there is currently no method to accurately monitor trilostane treatment, the plasma cortisol concentration obtained 3-hours posttrilostane administration was considered adequate (1.6 μg/dL).[14] Therefore, because systolic hypertension, nervousness and agitation remained despite adequate CS control, the possibility of concurrent pheochromocytoma was considered. Accordingly, urinary vanillylmandelic acid/creatinine ratio (VMA/Cr) was requested as diagnostic screening for pheochromocytoma. The VMA/Cr ratio was increased (271×10^{-3}; RI, $<58.2 \times 10^{-3}$), supporting the diagnosis of concurrent pheochromocytoma. Because left adrenalectomy was not authorized by the owner, instructions were given to administer antihypertensive treatment with

doxazosin mesylate at a dosage of 0.5 mg/kg PO q24h indefinitely (Alpha-1-adrenoceptor antagonist; Cardura XL, Pfizer, New York, New York). Two weeks later, the dog died as a result of a cardiorespiratory crisis characterized by cardiac arrhythmias (not specified in the record), hypertension, tachypnea, and pulmonary edema.

At necropsy, the pituitary gland was found in a normal intrasellar position (below the dorso-sellar process) with a small superficial cyst in the cranial region of the adenohypophysis (Figure 1A,B). Both adrenal glands were enlarged (left adrenal gland, 21 × 14 × 13 mm; right adrenal gland, 27 × 12 × 10 mm [length × width × height, respectively]) with multiple 1 to 4 mm internal yellowish nodules, but the left adrenal gland was slightly deformed by an internal well-defined round reddish tumor 11 mm in diameter (Figure 2A,B). No other neoplasms were found at necropsy.

The pituitary gland and both adrenal glands were fixed in 10% neutral buffered formalin. Once fixed, the pituitary gland was cut sagittally into 2 halves which were embedded in paraffin and, 4-μm-thick histological sections were cut from each half. Sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), reticulin stain, and ACTH immunostaining (monoclonal rabbit antihuman ACTH [EP390], Cell Marque, 1/750) to detect areas of corticotropic hyperplasia or a corticotropic adenoma.[15,16] Corticotropinoma was ruled out with HE, PAS and ACTH staining (Figure 3A-C), whereas reticulin network staining was not compatible with hyperplasia. Adrenal sections (4-μm-thick) also were stained with HE and subsequently incubated with primary antibodies for synaptophysin (monoclonal mouse antihuman synaptophysin [sc-55 507], Santa Cruz Biotechnology, 1/50) and 3βHSD2 (polyclonal rabbit antihuman 3βHSD2 [15516], Proteintech, 1/100) to characterize the origin (cortical or medullary) of the left adrenal neoplasm. A proliferation of polyhedral cells with a neuroendocrine appearance (scant cytoplasm with fine acidophilic granules) in a solid nesting pattern was found in the medulla of the left adrenal gland. The tumor was poorly defined with an infiltrative effect on the adrenal cortex, with 10 to 40 mitoses per 400× field and evidence of medullary tumor emboli. Immunohistochemistry of the neoplasm was positive for synaptophysin and negative for 3βHSD, consistent with a pheochromocytoma (Figure 4). No other areas of immunostaining were present in the adrenal cortex for synaptophysin.

In both adrenal cortices, a proliferation of 3 cell populations was found with a mitotic index of <1 per 400× field. The first population consisted of large clear hyperplastic cells (microvacuolated cytoplasm and central nucleus) located in the fascicular and reticular zones that were arranged in trabeculae and pseudoacini (human spongiotic cell-like). Among these, a second cell population arranged in multiple cell clusters with small nuclei, generally eccentric and with large microvacuolated cytoplasm, was detected. The third population was an accumulation of acidophilic polyhedral cells (defined borders, ample granular cytoplasm with occasional microvacuoles and round and irregular nucleus), partially circumscribed, not encapsulated, located at the junction of the fascicular and glomerular zones (human compact cell-like).[17]

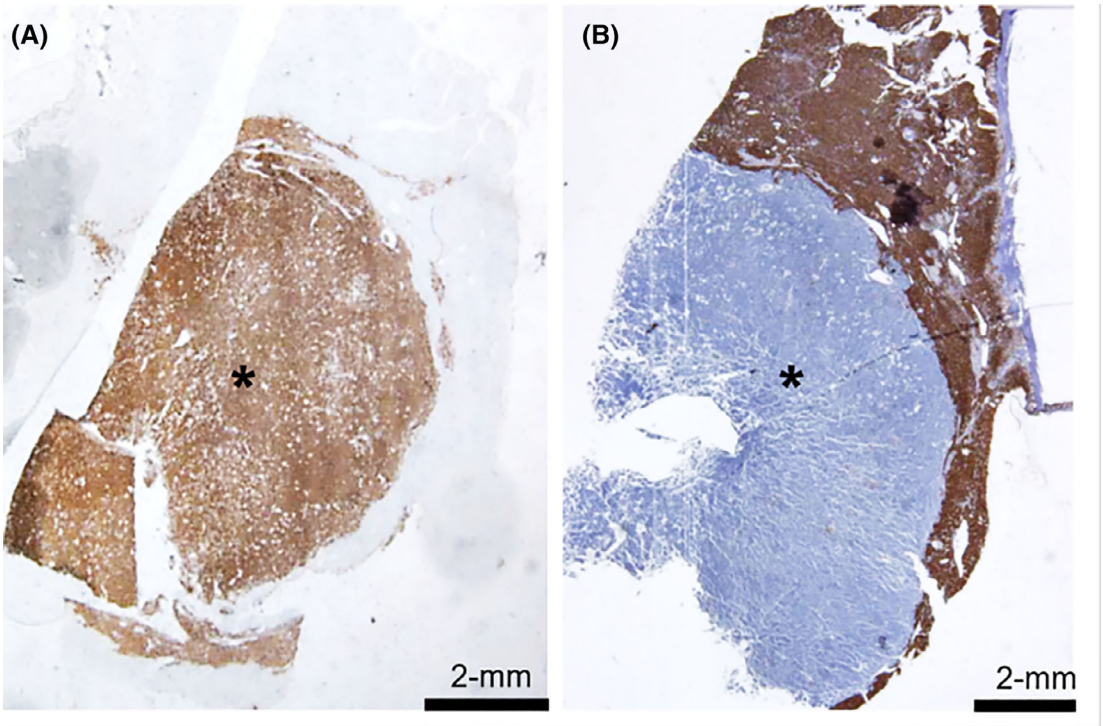

FIGURE 4 Immunohistochemical detection of synaptophysin and 3beta-hydroxysteroid dehydrogenase in the canine left adrenal gland. (A) Synaptophysin immunoreactivity was visualized brown in the pheochromocytoma (asterisk) but not in the surrounding adrenal cortex. (B) Reciprocally, 3beta-hydroxysteroid dehydrogenase immunoreactivity was detected (brown color) in the adrenal cortex but not in the pheochromocytoma (asterisk)

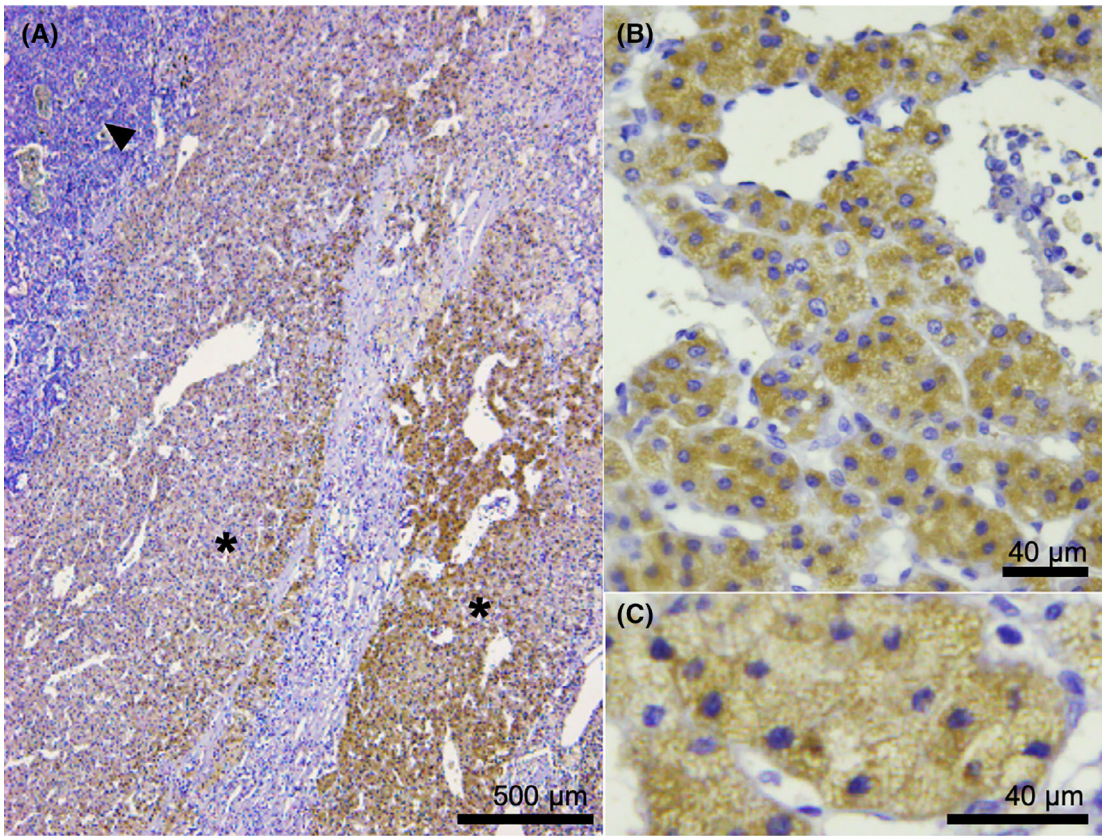

FIGURE 5 Immunohistochemical detection of adrenocorticotropic hormone (ACTH) in the canine left adrenal gland. ACTH immunoreactivity (brown) was visualized in groups of adrenocortical cells at the zona glomerulosa/zona fasciculata junction (asterisk), but not in the pheochromocytoma tissue (black arrowhead) (A). (B and C) Higher magnification views showing adrenocortical ACTH-positive cells. These cells included both compact and lipid-loaded spongiocytic cells

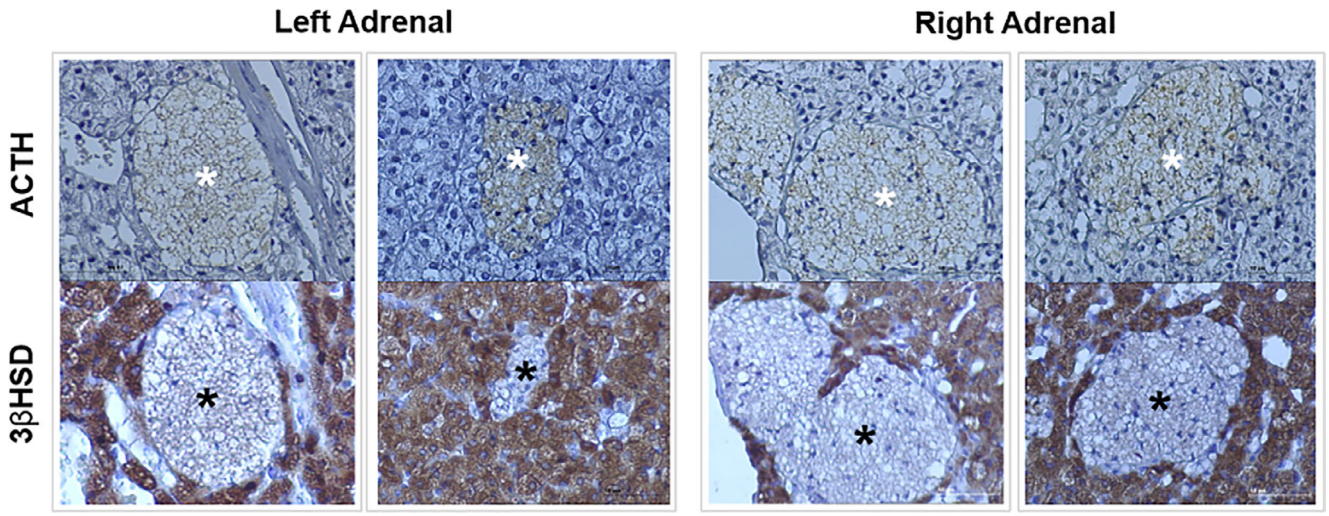

FIGURE 6 Detection of clusters of ACTH-positive spongiotic-like cells (white asterisks) appearing negative for 3beta-hydroxysteroid dehydrogenase (3βHSD) (black asterisks) in the cortex of the two adrenal glands

Immunohistochemical detection of ACTH was considered to indicate the diagnosis of ectopic secretion of corticotrophin by pheochromocytoma tissue as a cause of bilateral nodular adrenocortical hyperplasia and hypercortisolism. In addition, it appeared possible that, as in humans with PBMAH, production of ACTH by a subpopulation of corticosteroidogenic cells may have activated cortisol production through a paracrine mechanism.[18] Consistent with this hypothesis, ACTH immunoreactivity was not detected in the pheochromocytoma tissue but could be visualized in

compact cells (Figure 5) and clusters of large spongiocytic cells of both adrenal cortices (Figure 6). Only ACTH-positive compact cells expressed 3βHSD. Furthermore, paraffin-embedded pituitary tissue that had not been processed initially (both halves) was cut at 4-μm-thickness until all tissue was sectioned, and every 100-μm-interval, histologic sections were taken for HE, PAS, reticulin and ACTH. No proliferative lesions were found in the sections taken from each half. Additionally, negative immunoreactivity for ACTH was found in adrenal tissue from healthy, CS and pheochromocytoma dogs. Materials and methods, and immunohistochemistry images of adrenal controls are found in Data S1 and Figure S1, respectively.

3 | DISCUSSION

This report describes a dog with primary bilateral nodular adrenocortical hyperplasia associated with a pheochromocytoma. Currently, this association is referred to in the veterinary literature as concurrent endocrine neoplasia.[19] However, clinical and hormonal aspects that could suggest the presence of CS were not reported.

ACTH-dependent CS in dogs classically results from a functional pituitary adenoma (corticotropinoma) or, exceptionally, from a neuroendocrine tumor (ectopic ACTH syndrome).[1] In humans, in addition to the previously mentioned etiologies, pheochromocytoma and PBMAH also have been reported as the cause of clinical or subclinical CS caused by ectopic ACTH production.[17] To our knowledge, apart from a recent report of a case with pheochromocytoma, PBMAH has not been reported to cause CS by ectopic ACTH production in dogs.[5]

In our case, ectopic ACTH secretion by pheochromocytoma was ruled out as a recognized cause of bilateral adrenal hyperplasia and CS in humans, based on the lack of ACTH immunoreactivity in the tumor tissue together with suppressed plasma ACTH concentration.[20] In fact, although negative immunoreactivity is possible in both pheochromocytoma and ACTH-producing neuroendocrine tumors, the plasma concentration of ACTH in ectopic ACTH syndrome typically is increased.[2-5,20] Conversely, pituitary ACTH is suppressed by increased cortisol, which is stimulated by the paracrine action of intra-adrenocortical ACTH in humans with PBMAH and CS.[18] In the present case, as in PBMAH caused by intra-adrenocortical ACTH, circulating ACTH was not detected. A small adenoma or a focus of hyperplasia of the corticotropic cells could have been missed in the histological evaluation of the pituitary gland. However, the absence of focal PAS staining and focal immunostaining for ACTH in multiple sections, together with the absence of alterations in the reticulin network, ruled out this possibility in the dog of this report.[15,16] Moreover, the suppressed plasma ACTH concentration together with the absence of histological alterations in the pituitary gland and absence of neoplasms at necropsy, made it possible to dismiss pituitary and ectopic origins of CS in this case.

Previous case reports confirmed the expression, secretion and paracrine action of ACTH on PBMAH in humans with clinical or subclinical CS.[9-12] Surprisingly, these patients have low or suppressed ACTH concentrations, suggesting that not all forms of ACTH-dependent adrenal hyperplasia have increased circulating ACTH concentrations.[11] This report describes the first case of CS in a dog caused by primary bilateral nodular adrenocortical hyperplasia associated with abnormal expression of intra-adrenocortical ACTH. In veterinary medicine, primary or adrenal CS (ACTH-independent), without an adrenal tumor, but with bilateral adrenal enlargement, based on tomography or ultrasound, has only been described in 1 dog, possibly as a result of aberrant receptors for GIP (food-dependent hypercortisolism). However, the histological evaluation of adrenal glands was not documented by the authors.[7]

Immunostaining for ACTH was present in the adrenal cortex in our case as it occurs in humans with PBMAH.[9-12] Although both adrenal cortices had a macroscopic micronodular appearance, the histological evaluation was compatible with diffuse hyperplasia. This apparent discrepancy between the micronodular macroscopic appearance of the adrenal glands and the pattern of diffuse hyperplasia observed histologically could be explained by a lack of atrophy of the internodular adrenocortical tissue.[17] Bilateral diffuse adrenocortical hyperplasia is the main finding in dogs with ACTH-dependent CS, but in some of these the hyperplasia may be nodular.[6] Bilateral nodular adrenocortical hyperplasia is not common in dogs, being mainly a necropsy finding in older dogs.[21] Nonetheless, currently, there are no studies establishing the association with clinical signs, hormonal test results, and histological evaluation of the pituitary gland and CS.

Two main types of ACTH-positive steroidogenic cells have been described in humans with PBMAH: large cells filled with lipid droplets called clear cells or spongiocytic cells, and small dense eosinophilic cells called compact cells.[9-12] In our dog, 1 cell type was similar to human compact cells with positive staining for ACTH and a second type, similar to human spongiocytic cells positive for ACTH and negative for 3βHSD. Although colabelling with ACTH and 3βHSD antibodies was not confirmed in compact cells, it appears that the areas of the adrenal tissues positive for ACTH also expressed 3βHSD but not synaptophysin, indicating that they likely correspond to adrenocortical tissue. In this regard, it is also noteworthy that ACTH-positive 3βHSD-negative cells displayed the morphological appearance of steroidogenic cells. This finding raises the possibility that, as in human tissues, steroidogenic enzymes are differentially expressed in the 2 main cell types (compact and large clear cells) that compose macronodular adrenal hyperplasia.[22] However, it also seems that there are some species specificities because, in adrenal hyperplasia in humans, spongiotic cells are positive for 3βHSD, whereas compact cells are negative.[22] For technical reasons, the origin of ACTH-positive cells could not be determined, but, as in humans, these cells could belong to gonadal lineage, and their growth could be induced by the effect of luteinizing hormone as has been shown in postmenopausal women or castrated ferrets.[10,23,24]

Unlike humans, in which CS caused by PBMAH frequently is associated with subclinical cortisol secretion, the clinical presentation and hormonal test results were consistent with typical CS in the dog of our report.[17,25] Initially, the suppressed ACTH concentration associated with an adrenal tumor was wrongly interpreted as ACTH-independent CS caused by a cortisol-secreting adrenocortical tumor. However, as a result of the enlargement of the contralateral adrenal

gland instead of atrophy, a concurrent corticotropinoma, an ectopic ACTH syndrome or bilateral cortisol-secreting adrenocortical tumor were considered. Corticotropinoma and ectopic ACTH syndrome were excluded as previously discussed. Bilateral adrenocortical neoplasia, although difficult to differentiate from bilateral hyperplasia, was ruled out based on the size and shape of the adrenal glands and histological characteristic of both adrenal cortex (low mitotic index, noncapsular invasion, and absence of vascularization).[26]

Immunoreactivity for ACTH was detected in adrenocortical tissue in our case and was associated with CS, suggesting that locally produced corticotropin was responsible for increased cortisol secretion. However, the ultimate demonstration that intra-adrenal ACTH actually was produced by the hyperplastic tissue would have necessitated in vitro studies using cultured adrenocortical cells and detection of ACTH in culture medium.

In conclusion, based on the present case, it can be inferred that CS also may occur as a consequence of primary bilateral nodular adrenocortical hyperplasia in dogs. Likewise, as in humans with PBMAH, hypersecretion of cortisol may result from ectopic synthesis of ACTH in adrenocortical cells. Hypercortisolism inhibits pituitary ACTH production and consequently suppresses plasma ACTH concentration. Therefore, the term ACTH-independent used to designate primary adrenal CS may be inappropriate in some cases of bilateral adrenocortical hyperplasia in the absence of pituitary or neuroendocrine tumors in dogs.

ACKNOWLEDGMENT

This work was partially supported by the University of Buenos Aires-UBACyT (code: 20720170100006BA; 2018-2021), Argentina. We thank Florencia Trubbo (histotechnician) for the histological procedures, and Drs Carolina Vecino and Angelica Kim for the preliminary histological evaluations.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires approved all procedures (Protocol CICUAL #2013/17 and UBACyT 20720170100006BA), and written consent was obtained from the proprietor for carrying out all evaluations and their subsequent publication.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

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How to cite this article: Soler Arias EA, Castillo VA, Louiset E, Lefebvre H. Cushing's syndrome caused by intra-adrenocortical adrenocorticotropic hormone in a dog. *J Vet Intern Med*. 2022;36(1):264-271. doi:10.1111/jvim.16320