



## SPONTANEOUSLY ARISING DISEASE

# Presence of CD3<sup>+</sup> and CD79a<sup>+</sup> Lymphocytes in the Pituitary Gland of Dogs at Post-mortem Examination

M. A. Blomqvist<sup>\*</sup>, C. Ley<sup>†</sup>, H. K. Karlsson<sup>†</sup> and J. M. Hanson<sup>\*</sup>

<sup>\*</sup> Department of Clinical Sciences and <sup>†</sup> Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden

## Summary

Hypophysitis has been reported occasionally in dogs, with most cases resembling primary lymphocytic hypophysitis in man. Although it is generally assumed that lymphocytes are not present normally in the canine pituitary gland, few studies have investigated this hypothesis. However, lymphocytes are recognized in the pituitary gland of people and horses without signs of pituitary disease. It is unknown to what degree lymphocyte infiltration of the pituitary gland might occur as an incidental finding in dogs. The aim of the present study was to investigate the presence and distribution of lymphocytes in the pituitary gland of dogs without clinical suspicion of pituitary disease. Twenty dogs were subjected to routine necropsy examination. Formalin-fixed and paraffin wax-embedded sections of pituitary were stained with haematoxylin and eosin (HE) or subjected to immunohistochemistry (IHC) using primary antibodies specific for the T-cell marker CD3 and the B-cell marker CD79a. The number of CD3<sup>+</sup> and CD79a<sup>+</sup> cells per area unit (CPA) was determined for different pituitary regions. Two dogs had extensive neoplastic lesions in the pituitary gland and were excluded from analysis. In the remaining 18 dogs, occasional scattered CD3<sup>+</sup> cells were found in the pituitary gland. There was a significant difference in CD3<sup>+</sup> CPA between pituitary regions ( $P = 0.001$ ). The highest CD3<sup>+</sup> CPA was found in the pars tuberalis (median 41.3 cells/mm<sup>2</sup>, interquartile range 20.9–50.5 cells/mm<sup>2</sup>). In six of the 18 dogs (33%), CD79a<sup>+</sup> cells were detected in small number (median total cell number 0 cells/section, interquartile range 0–1.0 cells/section). This study shows that T cell, and fewer B cells, may be found in the pituitary gland of dogs without clinical suspicion of pituitary disease. Regional difference in T-cell density, with the highest CD3<sup>+</sup> CPA in the pars tuberalis, may imply regional immunoregulatory functions in the canine pituitary gland.

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Inflammation of the pituitary gland (hypophysitis) is recognized increasingly in man, and there are cases reported in dogs and other animal species (McAllister, 1991; Adissu *et al.*, 2010; Cramer *et al.*, 2011; Wolfesberger *et al.*, 2011; Meij *et al.*, 2012; Caturegli *et al.*, 2014; Rudinsky *et al.*, 2015; Grau-Roma *et al.*, 2017; Polledo *et al.*, 2017, 2018; Miller *et al.*, 2018). Hypophysitis may result in pituitary

enlargement and dysfunction (Caturegli *et al.*, 2005). Symptoms and clinical signs in affected people are mainly associated with the compression of surrounding tissue and hypopituitarism (Caturegli *et al.*, 2005; Hanna *et al.*, 2015). The most common underlying cause is primary lymphocytic hypophysitis, thought to have an immune-mediated aetiology (Caturegli *et al.*, 2005, 2014). On histopathology, the inflammatory infiltrate typically comprises lymphocytes, plasma cells and occasional

Correspondence to: J. M. Hanson (e-mail: [jeanette.hanson@slu.se](mailto:jeanette.hanson@slu.se)).

myeloid cells (Caturegli *et al.*, 2005). Of the 12 dogs reported previously with hypophysitis, seven (58%) had primary lymphocytic hypophysitis (Supplementary Table 1).

The presence of diffuse lymphocytic infiltration of the pituitary gland at autopsy is a common incidental finding in man (Simonds and Brandes, 1925; Shanklin, 1951). Recently, lymphocytes were detected in 50% (12 of 24) of the pituitary glands of horses at necropsy examination, when sections were stained with haematoxylin and eosin (HE). With immunohistochemistry (IHC), T lymphocytes were detected in all horses ( $n = 16$ ) (Grau-Roma *et al.*, 2017). Although there is a paucity of studies on the presence of lymphocytes in the pituitary gland in dogs, it is generally assumed that lymphocytes are not normally present. The aim of this study was to investigate the presence and regional distribution of lymphocytes in the pituitary glands of dogs without clinical suspicion of pituitary disease.

With a cross-sectional study design, pituitary glands were collected from 20 dogs, older than 12 months and without clinical suspicion of pituitary disease, at the time of routine necropsy examination at the Section of Pathology, Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden, from November 2013 to February 2014 and April 2017 to September 2017. The pituitary glands were collected *en bloc* with the hypothalamic connection intact, fixed in 10% neutral buffered formalin for 1–4 days, sectioned sagittally, processed routinely and embedded in paraffin wax. Sections (4  $\mu\text{m}$ ) were stained with HE and subjected to IHC. For IHC, sections were dewaxed in xylene and rehydrated in descending graded ethanols (100%, 95% and 70%) and distilled water. For antigen retrieval, sections were incubated in tris-ethylenediamine tetra-acetic acid (tris-EDTA) buffer (pH 9.0) at 92°C for 20 min. Sections were washed in tris-buffered saline with 0.1% polysorbate 20 (TBST) buffer and immunolabelled with monoclonal mouse anti-human primary antibodies specific for the T-cell marker CD3 (clone F7.2.38, M7254; Dako, Glostrup, Denmark; dilution 1 in 400 and 1 in 800) and the B-cell marker CD79a (clone HM57, M7051; Dako; dilution 1 in 200). For both primary antibodies, the reactivity and specificity for the targeted antigens was demonstrated (Furukawa *et al.*, 2017). The binding of antigen and antibody was ‘visualized’ using an avidin–biotin–peroxidase system (Vectastain Elite ABC HRP Kit, PK-6102; Vector Laboratories, Burlingame, California, USA) and 3, 3'-diaminobenzidine (DAB; ImmPACT DAB Peroxidase Substrate, SK-4105; Vector Laboratories)

as chromogen. Solutions were prepared according to the manufacturer's instructions, with modification of the secondary antibody solution that was diluted to 1 in 800 with 1% dog serum added in order to block non-specific binding. Endogenous peroxidase activity was blocked with 3%  $\text{H}_2\text{O}_2$  at room temperature for 5 min. The sections were counterstained with haematoxylin, dehydrated in ascending graded ethanols (70%, 95% and 100%), cleared in xylene and mounted with cover slips. Sections from a lymph node of one of the included dogs was used as positive control. As negative control, pituitary sections from each dog were used with the primary antibody omitted.

The HE-stained sections were evaluated with light microscopy (Olympus BH-2 Microscope; Olympus, Tokyo, Japan). Morphological changes, including the presence of lymphocytes, were recorded and categorized into three different groups: 1, no lesions; 2, limited lesions (i.e. cyst-like changes and nodular hyperplasia); or 3, extensive lesions (i.e. neoplastic lesions with impact on pituitary architecture). In IHC, the number of positively labelled CD3 ( $\text{CD3}^+$ ) and CD79a ( $\text{CD79a}^+$ ) expressing cells per area unit (CPA) was determined, with a microscope eyepiece with counting grid reticule at  $\times 400$  magnification, for different regions of the pituitary gland: the pars tuberalis; pars distalis; pars intermedia; infundibulum and pars nervosa (Supplementary Fig. 1). The area of the regions ranged from 0 to 2.0  $\text{mm}^2$ . Criteria for  $\text{CD3}^+$  and  $\text{CD79a}^+$  cells were morphology consistent with lymphocytes (i.e. small cell with round to slightly indented nucleus and sparse cytoplasm) and the nucleus encircled by brown chromogen-positive cytoplasm. Intravascular and intrasinusoidal  $\text{CD3}^+$  and  $\text{CD79a}^+$  cells were excluded from recording.

Inferential analysis was performed on data retrieved from CD3-labelled sections from dogs with no or limited lesions in their pituitary glands. Dogs with extensive lesions were excluded from inferential analysis because of the general loss of normal pituitary architecture. Non-parametric tests were used, based on non-Gaussian distributions, small sample size and heteroscedasticity in residual plots generated from initial testing, using mixed effect analysis, of the relationship between  $\text{CD3}^+$  CPA and the pituitary region. Differences in  $\text{CD3}^+$  CPA between different pituitary regions was assessed by use of the Skillings–Mack test. Each dog (section) was considered as a block. Post-hoc analysis was performed by multiple pairwise comparisons using the Wilcoxon signed-rank test with Holm–Bonferroni correction for multiple comparisons. Spearman's rank correlation tests with Holm–Bonferroni correction for multiple comparisons were performed for each pituitary

region with age and CD3<sup>+</sup> CPA as variables. Statistical analysis was performed using R software (version 3.5.2, R Foundation for Statistical Computing, Vienna, Austria, 2018) and packages: lme4 (version 1.1-19, Bates D, Maechler M, Bolker B, Walker S, 2018) and Skillings.Mack (version 1.10, Srisuradetchai, 2015). The significance threshold was set at 0.05.

The median age of the 20 dogs was 8.3 years (interquartile range 6.9–9.6 years). There were 17 female dogs (85%) and three male dogs (15%), of which, respectively, five (29%) and one (33%) were neutered. There were 14 different breeds and one mixed breed dog (5%) (Supplementary Table 2). The most common necropsy diagnosis was multicentric lymphoma, reported in four dogs (20%), followed by osteoarthritis reported in three dogs (15%) (Supplementary Table 3). The morphological changes recorded in HE-stained sections of the pituitary glands are summarized in Table 1. No lymphocytes were detected in HE-stained sections in the 18 dogs (90%) with no or limited lesions in their pituitary glands.

With IHC, low numbers of scattered CD3<sup>+</sup> cells were found in the pituitary glands of all 18 dogs (90%) with no or limited lesions in their pituitary glands (Fig. 1, Supplementary Fig. 2). There was a significant difference in CD3<sup>+</sup> CPA between different regions of the pituitary gland ( $\chi^2_4 = 45.9, P < 0.001$ ) (Fig. 2). The highest CD3<sup>+</sup> CPA was found in the pars tuberalis (median 41.3 cells/mm<sup>2</sup>, interquartile range 20.9–50.5 cells/mm<sup>2</sup>). The lowest CD3<sup>+</sup> CPA was found in the infundibulum (median 2.0 cells/mm<sup>2</sup>, interquartile range, 0–6.0 cells/mm<sup>2</sup>). On post-hoc pairwise comparisons, the CD3<sup>+</sup> CPA was significantly higher in the pars tuberalis compared with the pars distalis ( $P = 0.003$ ), pars intermedia ( $P = 0.004$ ), infundibulum ( $P = 0.003$ ) and the pars nervosa ( $P = 0.005$ ). The CD3<sup>+</sup> CPA in the pars distalis was significantly higher than in the infundibulum ( $P = 0.004$ ). There was no correlation between age and CD3<sup>+</sup> CPA in any of the pituitary regions, independent of the use of uncorrected or adjusted  $P$  values for multiple comparisons.

Cells positive for CD79a on IHC were detected in small number (median total cell number 0 cells/section, interquartile range 0–1.0 cells/section) in the pituitary glands of six of the 18 dogs (33%) with no or limited lesions in their pituitary glands. A heavy infiltration of CD79a<sup>+</sup> cells (>1,500 cells/mm<sup>2</sup> in the pars tuberalis, pars distalis and pars intermedia, and 300 cells/mm<sup>2</sup> in the infundibulum and pars nervosa) was observed in the pituitary gland of one dog with multicentric lymphoma and this was interpreted as a neoplastic infiltration. Although the neoplastic lymphocytic infiltrate was composed mainly of

**Table 1**  
**Morphological changes recorded in the pituitary glands from the 20 dogs of the study**

Lesion classification	Notes	n*	%†
<b>Extensive lesion</b>			
Neoplastic lymphocytic infiltration	Heavy lymphocytic infiltration involving the entire pituitary gland in a dog with multicentric lymphoma	1	5
Pituitary adenoma	Well-defined neoplastic change, approximately 6 mm in diameter, consisting of a nodular proliferation of basophilic polygonal cells compressing and distorting the normal pituitary parenchyma	1	5
<b>Limited lesion</b>			
Cyst-like change	Cyst-like changes in the pars distalis showing varying presence of peripheral epithelial lining and central pale mucinous material	6	30
Nodular hyperplasia	Small, approximately 0.3 mm diameter, foci of nodular hyperplasia in the pars distalis and consisting in both cases of basophilic polygonal cells showing mild anisocytosis, mild anisokaryosis and no mitotic activity	2	10
<b>No lesion</b>			
		10	50

\*Number of dogs.

†Percentage of dogs.

CD79a<sup>+</sup> cells, a high number of CD3<sup>+</sup> CPA was found in the pars tuberalis (116 cells/mm<sup>2</sup>), pars distalis (168 cells/mm<sup>2</sup>), pars intermedia (66.7 cells/mm<sup>2</sup>) and pars nervosa (172 cells/mm<sup>2</sup>).

There was no noticeable increase in CD3<sup>+</sup> or CD79a<sup>+</sup> cells in the pituitary parenchyma adjacent to hyperplastic or cyst-like changes in the dogs included for analysis. However, the dog with a pituitary adenoma showed markedly higher CD3<sup>+</sup> CPA in the pars tuberalis (184 cells/mm<sup>2</sup>), pars distalis (60 cells/mm<sup>2</sup>) and infundibulum (52 cells/mm<sup>2</sup>) compared with the 18 dogs included for analysis (Fig. 3).

The present study showed that CD3<sup>+</sup> cells were common in the pituitary glands of middle-aged to elderly dogs without clinical suspicion of pituitary disease, and without lymphocytes being recognized on

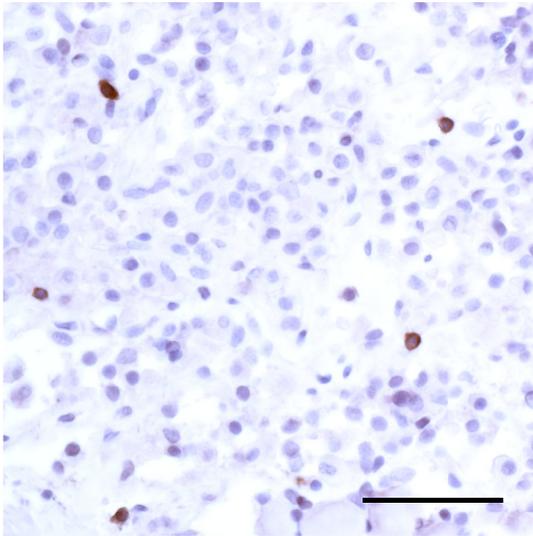


Fig. 1. Scattered CD3<sup>+</sup> cells in the pars distalis of a dog with no concurrent lesions in the pituitary gland. IHC. Bar, 50 μm.

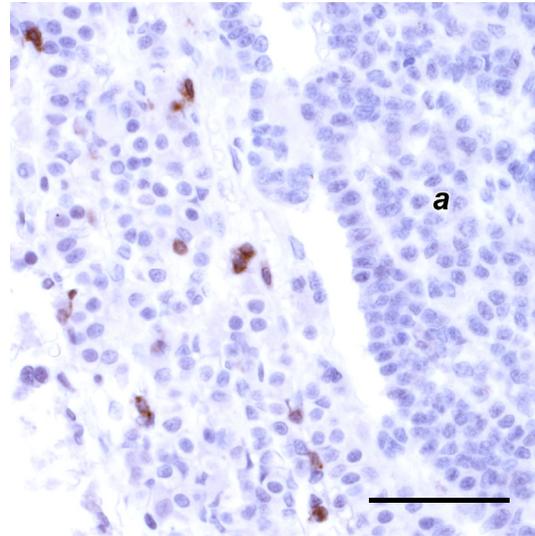


Fig. 3. CD3<sup>+</sup> cells in the pars distalis adjacent to a pituitary adenoma (a) in a dog. The number of CD3<sup>+</sup> cells per area unit was markedly higher compared with those in the 18 dogs with no or limited lesions in their pituitary glands. IHC. Bar, 50 μm.

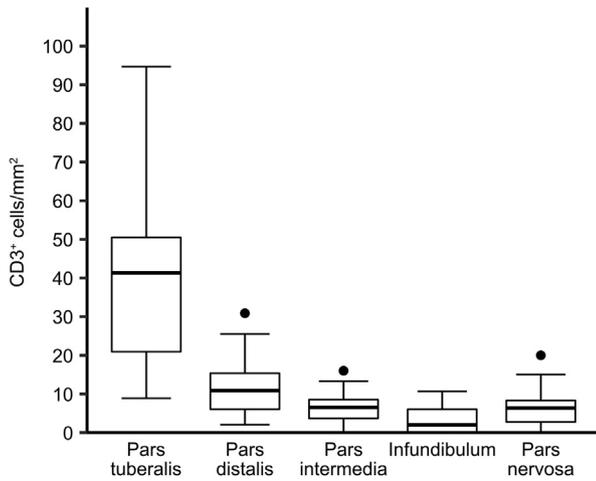


Fig. 2. Box plot showing the number of CD3<sup>+</sup> cells per area unit for different parts of the pituitary gland (pars tuberalis, pars distalis, pars intermedia, infundibulum and pars nervosa) at necropsy examination of 18 dogs without clinical suspicion of pituitary disease. Dots represent outliers.

routine HE-stained sections. The CD3<sup>+</sup> cell counts differed between regions. Less commonly, CD79a<sup>+</sup> cells were found.

The finding of CD3<sup>+</sup> cells in the pituitary glands of all dogs is consistent with previous findings in man and horses (Simonds and Brandes, 1925; Shanklin, 1951; Grau-Roma *et al.*, 2017). However, in contrast with those studies, no lymphocytes were recorded in HE-stained sections of the pituitary glands of the dogs in the present study, which may explain the previous assumption that lymphocytes are normally absent in the pituitary gland of dogs. In-

dividual lymphocytes can be difficult to differentiate from the nuclei-dense parenchymal cells of the adenohypophysis, especially if scattered diffusely. The findings of the present study therefore highlight the value of using IHC for lymphocyte detection in the pituitary gland.

There was a regional difference in CD3<sup>+</sup> cell density within the pituitary glands of the dogs. The highest CD3<sup>+</sup> CPA was found in the pars tuberalis, a well-vascularized site shown recently to have important regulatory function of the circannual biological rhythm (Lincoln, 2019).

In agreement with the findings in pituitary glands of horses, the majority of the dogs had no detectable CD79a<sup>+</sup> cells in the pituitary gland (Grau-Roma *et al.*, 2017). The observed proportions of CD3<sup>+</sup> cells, representing T cells, and CD79a<sup>+</sup> cells, representing B cells, in the pituitary gland may relate to physiological immune functions. T cells are the predominant circulating lymphocytes, which may explain why CD3<sup>+</sup> cells outnumbered CD79a<sup>+</sup> cells in the pituitary glands in the present study (García-Sancho *et al.*, 2014).

Interestingly, there was a high CD3<sup>+</sup> CPA in the pituitary parenchyma adjacent to a pituitary adenoma. It is possible that parenchymal compression and tissue distortion exerted by the lesion is an explanation for this finding. Alternatively, the CD3<sup>+</sup> cells may represent an immune response to the neoplastic lesion, which in many cases may help to limit neoplastic growth and progression (Dougan and

Dranoff, 2009). However, the presence of T cells in the microenvironment may have a stimulatory effect on corticotroph tumours, which in dogs have strong co-expression of the receptor for leukaemia inhibitory factor, a cytokine that is secreted by activated T cells (Hanson *et al.*, 2010; Metcalfe, 2011). In contrast, there was no increase in CD3<sup>+</sup> CPA in the pituitary parenchyma adjacent to small areas of nodular hyperplasia found in two dogs.

Limitations of the present study include a small sample size ( $n = 20$ ) and skewed sex distribution (17 female and three male dogs). Therefore, no attempt was made to compare differences between sexes. In addition, all samples were collected at necropsy examination. All dogs, even without specific clinical suspicion of disease, had concurrent diseases, which could have stimulated a lymphocytic reaction in the pituitary gland. Due to the presence of other diseases it can therefore only be concluded that it is common to find CD3<sup>+</sup> cells in the pituitary glands of dogs that have been humanely destroyed for reasons other than pituitary-related disease.

In conclusion, the present study shows that it is common to find T cells in the pituitary gland of dogs with no clinical suspicion of pituitary disease, which may represent a normal variation or subclinical hypophysitis, and less likely an age-related change, since there was no correlation between age and the number of CD3<sup>+</sup> cells. The predominance of T cells over B cells indicates that cellular immunological defence predominates over humoral defence in the pituitary glands of dogs. Regional differences in CD3<sup>+</sup> cell density may represent differences in T-cell immunological activity and response to neoplastic lesions.

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### Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jcpa.2020.02.010>.

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