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Occurrence of primary lymphocytic hypophysitis in two horses and presence of scattered T-lymphocytes in the normal equine pituitary gland

Llorenç Grau-Roma, Robert Peckham, Jacqui Paton, Anina Stahel, Simone de Brot[1]

School of Veterinary Medicine and Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, UK (Grau-Roma, de Brot); University College Dublin, Equine Veterinary Hospital, University College Dublin Campus, Belfield, Dublin, Ireland (Packham); Scarsdale Equine Veterinary Practice, Derby, UK (Paton); and Institute of Virology, University of Zurich, Vetsuisse Faculty, Zurich, Switzerland (Stahel).

[1]Corresponding author: Simone de Brot, School of Veterinary Medicine and Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK.
simone.debrot@nottingham.ac.uk

Running title: Lymphocytic infiltrates in the pituitary gland of horses

Abstract. The postmortem examination of a 14-year-old Appaloosa gelding with clinically diagnosed pituitary pars intermedia dysfunction showed a unique finding of moderate multifocal lymphocytic hypophysitis (LH). The pituitary glands of 24 horses submitted for postmortem examination were examined grossly and examined histologically for the presence of lymphocytes. Of these 23 horses, 1 additional case suffered from moderate LH. The 2 cases with LH tested negative for *Equid herpesvirus 1* and *4* by PCR and immunohistochemistry (IHC), and no viral particles were observed by electron microscopy in 1 case examined. The cause of LH remains unknown, but based on the T-lymphocytic nature of the inflammation and the human literature, an immune-mediated origin is hypothesized. In addition, the review of 24 cases revealed that 10 horses had few and small multifocal lymphocytic infiltrates within the pituitary gland; the remaining 12 horses showed no evident lymphocytes when examined by H&E. IHC for CD3 showed the presence of a small number of individual T-lymphocytes scattered through the gland in all examined horses, which appears therefore to be a normal feature of the pituitary gland in horses.

Key words: Equine; hypophysitis; pituitary gland; pituitary pars intermedia dysfunction.

The most common pituitary gland abnormality in horses is associated with increased size and activity of the pars intermedia (PI), which is referred to as pituitary pars intermedia dysfunction (PPID; previously known as equine Cushing's disease).[14] PPID is considered to be a neurodegenerative disease with loss of inhibitory dopaminergic input that eventually leads to increased PI cell proliferation. Horses with PPID have an enlarged pituitary gland as the result of hyperplasia or benign neoplasia of the PI.[14] The primary cause of PPID has not yet been identified, and a multifactorial origin is suspected. The clinical signs of PPID are broad and include hirsutism, weight loss, change in mentation or behavior, secondary infections, endoparasitism, laminitis, polydipsia and polyuria, and sweating abnormalities.[14]

In April 2014, a 14-year-old Appaloosa gelding, with clinically diagnosed chronic laminitis, PPID, and recurrent airway obstruction (RAO), was submitted to the Veterinary Pathology Service of the University of Nottingham (Loughborough, UK) for postmortem examination (horse 1). The only gross abnormality was observed in the pituitary gland, which was moderately reduced in size. This finding was unexpected given the clinical suspicion of PPID. On histologic examination, chronic laminitis and RAO were confirmed. The pituitary gland had moderate multifocal, often perivascular, lymphocytic inflammation in the pars nervosa (PN) with mild involvement of the PI, which was diagnosed as lymphocytic hypophysitis (LH). Uncertain of the significance of the observed LH, 23 more equine pituitary glands were collected and examined for gross and microscopic abnormalities.

Pituitary glands were collected from 23 randomly selected horse and pony autopsy cases performed between April 2014 and October 2015 (Table 1). The case collection includes equids of different breeds, sex, and age (1–28 years). Including horse 1, 24 pituitary glands were assessed grossly and histologically by 2 ECVP board-certified veterinary pathologists. Owner's

consent was obtained prior to submission of the horses for postmortem examination. Histologic assessment was done blindly. For those samples for which there was no agreement, a consensus was reached after re-examining and discussing the cases. Of each pituitary gland, 1–3 sagittal sections were cut and routinely processed for histology and stained with hematoxylin and eosin (H&E). The tissue was assessed for the presence of lymphocytes, and the animals were classified into group 1 (moderate lymphocytic infiltrates), group 2 (mild lymphocytic infiltrates), or group 3 (no evident lymphocytes). Additionally, the glands were assessed for other non-inflammatory histologic features (i.e., the presence of colloid-filled dilated follicles in the PI, PI hyperplasia, and intracytoplasmic brown granular pigment in pituicytes). These features were semi-quantified, in order to assess a potential association of those lesions with the lymphocytic infiltrates (Table 2). Several sections of brain from all cases were thoroughly examined histologically.

To further characterize the pituitary lymphocytic inflammation, immunohistochemistry (IHC) for CD3 (rabbit polyclonal anti-human,[a] dilution 1:400) and CD79a (mouse monoclonal anti-human,[b] dilution 1:400) was performed in 2 of 2 horses from group 1, 9 of 10 horses from group 2 (excluding horse 12 with PI adenoma), and 5 of 10 horses from group 3 (Table 2). The 5 horses from group 3 were selected with the aim of including a broad range of ages (1–15 years old). In order to rule out *Equid herpesvirus* (EHV) infection, an IHC for EHV-1 (rabbit polyclonal anti-equine,[c] dilution 1:750) was performed on formalin-fixed, paraffin-embedded (FFPE) pituitary gland tissue from horses 1–3. Following previously reported protocols, a multiplex real-time polymerase chain reaction (PCR) for the detection of EHV-1[5] and -4[6] was performed on FFPE pituitary gland tissue from horses 1–3.

Pituitary gland tissue of horse 1 was examined by transmission electron microscopy (TEM). FFPE tissue was deparaffinized and post-fixed in 2.5% glutaraldehyde, osmium tetroxide, and uranyl acetate, and then embedded in resin. Ultrathin (70 nm) sections were then contrasted with lead citrate and scanned thoroughly.

Statistical software[d] was used for statistical analysis. The distribution of the age variable was assessed by the Shapiro–Wilk test. Kruskal–Wallis and Mann–Whitney tests were used to compare means of age between groups. Fisher exact test was used to compare the proportion of animals from each sex category and the proportion of animals showing presence and absence of dilated follicles in PI, PI hyperplasia, and pigment in pituicytes between the groups in pairs.

Four of 24 (17%) horses were submitted with clinical suspicion of PPID (Table 1), based on clinical signs and/or elevated basal plasma adrenocorticotropic hormone (ACTH) concentrations. ACTH levels were measured in 3 of the 4 suspected PPID cases, which were compared to seasonally adjusted reference values. Horse 1 had a mildly increased plasma ACTH level of 7.17 pmol/L (June 2012; reference: <6.38 pmol/L) 23 months before postmortem examination. In horse 3, a severely increased basal ACTH concentration of 41.36 pmol/L (reference: <6.38 pmol/L) was measured in April 2014, the day before the autopsy was performed. In horse 6, plasma ACTH levels were measured 4 times between September 2012 and March 2014. The initial ACTH concentration was markedly increased (20.32 pmol/L, September 2012; reference: <10.34 pmol/L). The 3 following ACTH measurements were normal (January 2013: 4.22 pmol/mL; June 2013: 4.93 pmol/L) to mildly increased (February 2014: 7.83 pmol/L), which were last measured 3 months before autopsy. Two of the 4 horses with clinically diagnosed PPID (horses 1 and 6) were intermittently treated with the synthetic dopamine-agonist

pergolide[e] (one 1-mg tablet orally, daily) over several months. In addition, horses 2 and 7 had a history of behavioral problems, including increased frequency of urination in the latter.

Grossly, 5 of 24 pituitary glands were mildly to moderately enlarged (Supplemental Figs. 1 and 2). The gland of horse 1 was reduced in size with a discoid shape (3 × 2.5 × 1 cm), leaving a partially empty sella turcica (Supplemental Fig. 3). The remaining 18 horses had grossly normal pituitary glands (Supplemental Fig. 4; Table 1). Of 24 horses, 2 horses (1 and 2) had moderate and multifocal lymphocytic infiltrates within the pituitary gland, and were classified as group 1 (lymphocytic hypophysitis). Ten horses (3–12) had mild multifocal lymphocytic infiltrates within the pituitary gland, and were classified as group 2.The remaining 12 horses had no evident lymphocytes when examined by H&E (group 3; Table 2). No other leukocytes (e.g., plasma cells, macrophages, or granulocytes) were observed within the studied pituitary glands. The 3 groups contained horses and/or ponies of different breeds, sex, and age (Table 1). The mean age was 9.5 (± standard deviation [SD] 6.4), 16.6 (± SD 10.8) and 8.4 (± SD 4.2) years in group 1, 2, and 3, respectively. The age variable was shown to be non-normally distributed (p = 0.031), and Kolmogorov–Smirnov test indicated that horses from group 2 were significantly older than group 3 ($p < 0.047$), although the Mann–Whitney test indicated only a statistical trend ($p = 0.135$). No other statistically significant differences were found.

In the 2 cases included in group 1, the lesions were considered sufficient to diagnose LH. The lymphocytic aggregates were multifocal, usually around small vessels, and mainly located within the PN, with fewer within the PI in both cases (Fig. 1) but also in the pars distalis (PD) in horse 2. These 2 horses showed no hyperplasia or adenoma within the PI (Table 2). In group 2, lymphocytic aggregates were observed in the PN in 10 of 11, the PI in 5 of 12, and the PD in 5 of 12 horses. Two horses (11 and 12) with mild lymphocytic infiltrates also had PI adenomatous

hyperplasia with a microadenoma and a PI adenoma, respectively. The adenoma occupied most of the PI, expanding into adjacent pituitary lobes, with no remaining PN tissue in the studied sections. Dilated follicles in the PI (Fig. 2) and pigment within pituicytes were commonly seen in all 3 groups, showing therefore no association with the presence of lymphocytes. PI hyperplasia was frequent in groups 2 (6 of 9 horses) and 3 (7 of 12 horses). No evidence of inflammation was present in any of the brain sections examined.

In all of the examined horses with lymphocytic infiltrates within the pituitary gland, most lymphocytes (>50% in the studied sections) were $CD3^+$, being therefore compatible with T-lymphocytes (Fig. 3). In 2 of 2 horses of group 1 and 2 of 9 horses of group 2, a very low number of lymphocytes were $CD79a^+$ (Fig. 4).The 5 horses studied from group 3, with no evident lymphocytic infiltrates on H&E, had a low number of individual $CD3^+$ lymphocytes, regularly scattered throughout the PN, PI, and PD (Fig. 5).

Horses 1 and 2 with LH and horse 3 from group 2 were tested for infection with EHV-1 and -4 by PCR and IHC, which gave negative results. In addition, horse 1 was also examined by TEM, which confirmed the presence of lymphocytes, but lacked evidence of viral particles (Fig. 6).

As far as we are aware, natural cases of primary hypophysitis in animals have only been reported in dogs,[1,4,13,15,20] and in a cat.[19] In humans, hypophysitis is a rare condition with frequencies of <1% of surgical pituitary cases.[8,10] Primary hypophysitis in humans comprises 3 distinct histomorphologic entities: lymphocytic (the most common), granulomatous, and xanthomatous.[8,10] It can be acute, subacute, or chronic, with symptoms compatible with those of a pituitary mass, and is included in the differential diagnosis in cases of pituitary dysfunction.[8,10] LH in people is reported to often lead to enlargement of the pituitary gland but, interestingly,

people with chronic LH often have pituitary atrophy.[8] Our horse 1 showed an apparently smaller pituitary gland, which may correspond to a similar situation, although no evidence of fibrosis, as described in humans, was observed histologically.[8] Horse 2 had no obvious gross variation in the size of the pituitary gland. However, slight changes in size of the pituitary glands are difficult to appreciate. The use of previously reported methods measuring height, weight, and PI area,[17,19] not performed in our study, would have minimized subjective evaluation. Histologically, the nature of the lesions in both horses with LH was similar to the ones reported in the human literature in cases of primary LH, consisting of multifocal lymphocytic aggregates, predominantly of T-lymphocytes with sparse numbers of B-lymphocytes.[9] However, although in humans the adenohypophysis is reported to be the area most affected, the main lesions in both LH-affected horses was in the PN, with only mild aggregates of lymphocytes present within the PD and/or PI. Moreover, although the presence of plasma cells is usually described in human cases, they were not present in our 2 horses.[9] No evidence of infectious agents were found in the brain or pituitary glands of the studied horses, indicating that the LH was primary.[10] IHC and PCR testing for EHV was negative in both horses, and examination of horse 1 by TEM failed to show any viral particles. Therefore, the cause of LH in both horses remains unclear. In humans, LH etiology is unclear, but is suspected to be autoimmune.[8] Based on the T-lymphocytic nature of the observed inflammation and the information available from humans, an immune-mediated origin is hypothesized for our equine cases.

Because of the novelty of the pituitary lesions, their detection in only 2 cases, and the limited clinical information available, it is difficult to correlate the LH with the clinical signs or other lesions observed in the postmortem examination. Horse 1 was submitted for autopsy with a clinical suspicion of PPID, based on mildly increased plasma levels of ACTH determined ~2

171 years before. However, the ACTH elevation must be interpreted cautiously because of the long
172 lapse of time between the last ACTH determination and the autopsy, the well-known ACTH
173 seasonal variations, and potential increases of ACTH concentration as the result of other factors
174 such as stress and/or pain.[2,3,7] In humans, ACTH concentrations are usually reduced in cases of
175 hypophysitis.[16] As well, ACTH concentrations were not measured in the second horse affected
176 with LH, and it did not have a clinical diagnosis of PPID. However, this horse was reported to
177 suffer from behavioral problems, one of the clinical signs reported with PPID.[14] Regarding other
178 lesions observed during postmortem examination, horse 1 had chronic laminitis, and horse 2
179 suffered from a chronic desmopathy; both conditions have been associated with PPID.[11,14]
180 Interestingly, however, neither of the 2 cases had adenoma or hyperplasia of the PI. In addition,
181 one of the horses was young (5 years), whereas the other was middle aged (14 years), suggesting
182 no relation with the observed lesion and aging.

183 Given the importance of the pituitary gland in horses, further studies including a large
184 number of horses with well-known clinical diagnosis (including PPID-affected horses and non-
185 affected horses with known ACTH concentrations) are needed to understand the clinical
186 importance as well as the frequency of occurrence of this condition. Current preliminary results
187 indicate that LH occasionally occurs in horses, and suggest that it may be worth reassessing a
188 large number of paraffin-embedded pituitary tissues for the presence of LH.

189 The presence of small aggregates of lymphocytes within the pituitary gland was a
190 frequent finding visible by H&E (10 horses [42%], group 2). These infiltrates were demonstrated
191 to consist of mainly T-lymphocytes by IHC. Three of the animals included in this group had a
192 clinical diagnosis of PPID. However, this diagnosis can be well explained due to the presence of
193 1 pituitary adenoma (horse 11) and hyperplasia of the PI (horse 3 and 6). Remarkably, in the

194 animals with no lymphocytic infiltrates evident on H&E (group 3), a low number of individual
195 T-lymphocytes were observed scattered through the pituitary parenchyma when using IHC. The
196 observation of aggregates of lymphocytes within the pituitary gland (group 2) and of individual
197 scattered T-lymphocytes (group 3) was similar regardless of sex and the presence of other
198 histologic changes in the pituitary gland. However, the animals within group 2 had a higher
199 mean age than the horses within group 3. These results suggest that the presence of a few
200 scattered T-lymphocytes within the equine pituitary gland is a normal finding, and that these may
201 form small aggregates visible on H&E with increasing age. As far as we are aware, the presence
202 of T-lymphocytes within normal pituitary glands has not been reported previously in horses but
203 is described as a normal finding in people.[12]

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209 **Authors' contributions**

210 L Grau-Roma and S de Brot contributed to conception and design of the study; contributed to
211 acquisition, analysis, and interpretation of data; drafted the manuscript; and gave final approval.
212 R Peckham contributed to conception of the study; contributed to acquisition, analysis, and
213 interpretation of data; and critically revised the manuscript. J Paton contributed to acquisition,
214 analysis, and interpretation of data, and critically revised the manuscript. A Stahel contributed to
215 analysis and interpretation of data, and critically revised the manuscript. All authors agreed to be

accountable for all aspects of the work in ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Sources and manufacturers

a. Dako UK Ltd., Cambridgeshire, UK.

b. Dako UK Ltd., Cambridgeshire, UK.

c. Produced in-house by Animal Health Trust, Newmarket, Suffolk, UK.

d. SPSS version 22, SPSS Inc., Chicago, IL.

e. Prascend (pergolide mesylate), Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table 1. Data and main autopsy findings from horses included in the study. Pituitary pars intermedia dysfunction (PPID) column indicates if clinical evidence of PPID was reported in the clinical history.*

Horse	Breed	Sex	Age (y)	PPID	Hypophysis gross size variation	Main autopsy diagnoses
Group 1						
1	Appaloosa	MN	14	Yes	Moderately reduced	Chronic laminitis, RAO
2	Arabian	FE	5	No†	Normal	Chronic suspensory and straight sesamoid desmopathy
Group 2						
3	Pony	MN	24	Yes	Moderately enlarged	Cholesteatoma
4	Cob	FE	1	No	Normal	Bronchopneumonia, cyathostomiasis
5	New Forest cross	MN	3	No	Normal	Pigmenturic nephrosis
6	Thoroughbred	MN	25	Yes	Slightly enlarged	Chronic laminitis, hirsutism
7	Thoroughbred	FE	8	No†	Normal	Acute interstitial pneumonia of unknown origin
8	Welsh pony cross	Male‡	24	No	Normal	Chronic perihepatitis, hepatic echinococcal cysts
9	Pony	MN	Adult	No	Normal	Malignant perineal melanoma with metastasis
10	Selle Français	Male‡	Adult	No	Normal	Degenerative hock joint disease
11	Pony	MN	28	Yes	Normal	Pituitary microadenoma, adrenal pheochromocytoma, thyroid adenoma
12	Irish draught	Male‡	20	No	Moderately enlarged	Pituitary adenoma, chronic laminitis
Group 3						
13	Knabstrupper	FE	10	No	Normal	Pyrrolizidine alkaloid toxicity, hepatic encephalopathy
14	“Sports horse”	ME	6	No	Slightly enlarged	Equine motor neuron disease
15	Welsh pony	MN	15	No	Normal	Chronic laminitis
16	Cob Tyle	Male‡	1	No	Normal	Emaciation, cyathostomiasis
17	Irish	MN	6	No	Normal	Rhinitis, lymphadenitis, enteritis
18	Thoroughbred	FE	14	No	Normal	Paralytic ileus after surgery
19	Trakehner × Morgan	MN	8	No	Normal	Grass sickness
20	Thoroughbred	FE	9	No	Normal	Chronic fibrous peritonitis
21	Pony	FE	6	No	Slightly enlarged	Chronic laminitis
22	Thoroughbred	MN	14	No	Normal	Radius fracture
23	Cob	FE	6	No	Normal	Suspected cerebellar degeneration
24	Warmblood	ME	6	No	Normal	Intestinal perforation due to foreign body

* FE = female entire; ME = male entire; MN = male neutered; RAO = recurrent airway obstruction.

† Behavioral problems reported in the clinical history.

‡ Neutering status not recorded.

Table 2. Histologic lesions within pituitary gland, and immunohistochemistry (IHC) for CD3 and CD79a.*

Horse	Lymphocytic infiltrates			Dilated follicles in PI	PI hyperplasia	Pigment in pituicytes	IHC	
	PD	PI	PN				CD3	CD79a
Group 1								
1	–	+	++	++	–	–	+++	+
2	+	+	++	+	–	++	+++	+
Total	1 of 2	2 of 2	2 of 2	2 of 2	0 of 2	1 of 2	2 of 2	2 of 2
Group 2								
3	–	–	+	++	+	++	+++	–
4	+	–	+	–	–	–	+++	–
5	–	–	+	–	–	+	+++	–
6	+	–	+	++	+	++	+++	–
7	+	+	+	–	–	+	+++	–
8	–	–	+	–	+	+	+++	–
9	–	+	–	+	+	+++	+++	–
10	–	–	+	+	+	–	+++	+
11	+	–	+	+	++	++	+++	+
12	–	+	NA†	–	NA‡	NA†	NP	NP
Total	4 of 10	3 of 10	8 of 9	5 of 10	6 of 9	7 of 9	9 of 9	2 of 9
Group 3								
13	–	–	–	+	+	+	+	–
14	–	–	–	+	+	++	NP	NP
15	–	–	–	+	+	+	+	–
16	–	–	–	–	–	–	+	–
17	–	–	–	–	–	–	NP	NP
18	–	–	–	–	–	+++	NP	NP
19	–	–	–	+	+	–	NP	NP
20	–	–	–	+	–	+	NP	NP
21	–	–	–	+	–	+	+	–
22	–	–	–	–	+	++	NP	NP
23	–	–	–	+	+	+	NP	NP
24	–	–	–	++	+	++	+	–
Total	0 of 12	0 of 12	0 of 12	8 of 12	7 of 12	9 of 12	5 of 5	0 of 5

* Lesions were semi-quantified as follows: – (absent), + (mild), ++ (moderate), and +++ (severe). For IHC, immunopositivity was semi-quantified as follows: – (negative), + (<10% of positive lymphocytes), ++ (10–50% positive lymphocytes), and +++ (>50% positive lymphocytes). Groups were defined based on the evaluation of H&E-stained slides as follows: group 1 (moderate lymphocytic infiltrates/lymphocytic hypophysitis), group 2 (mild lymphocytic infiltrates), and group 3 (no lymphocytic infiltrates). EHV = *Equid herpesvirus*; NA = not

294 applicable; NP = not performed; PD = pars distalis; PI = pars intermedia; PN = pars nervosa;

295 PPID = pituitary pars intermedia dysfunction, RAO = recurrent airway obstruction.

296 † No PN tissue observed in the studied sections.

297 ‡ The adenoma occupied most of the PI.

298

299

300

Figures

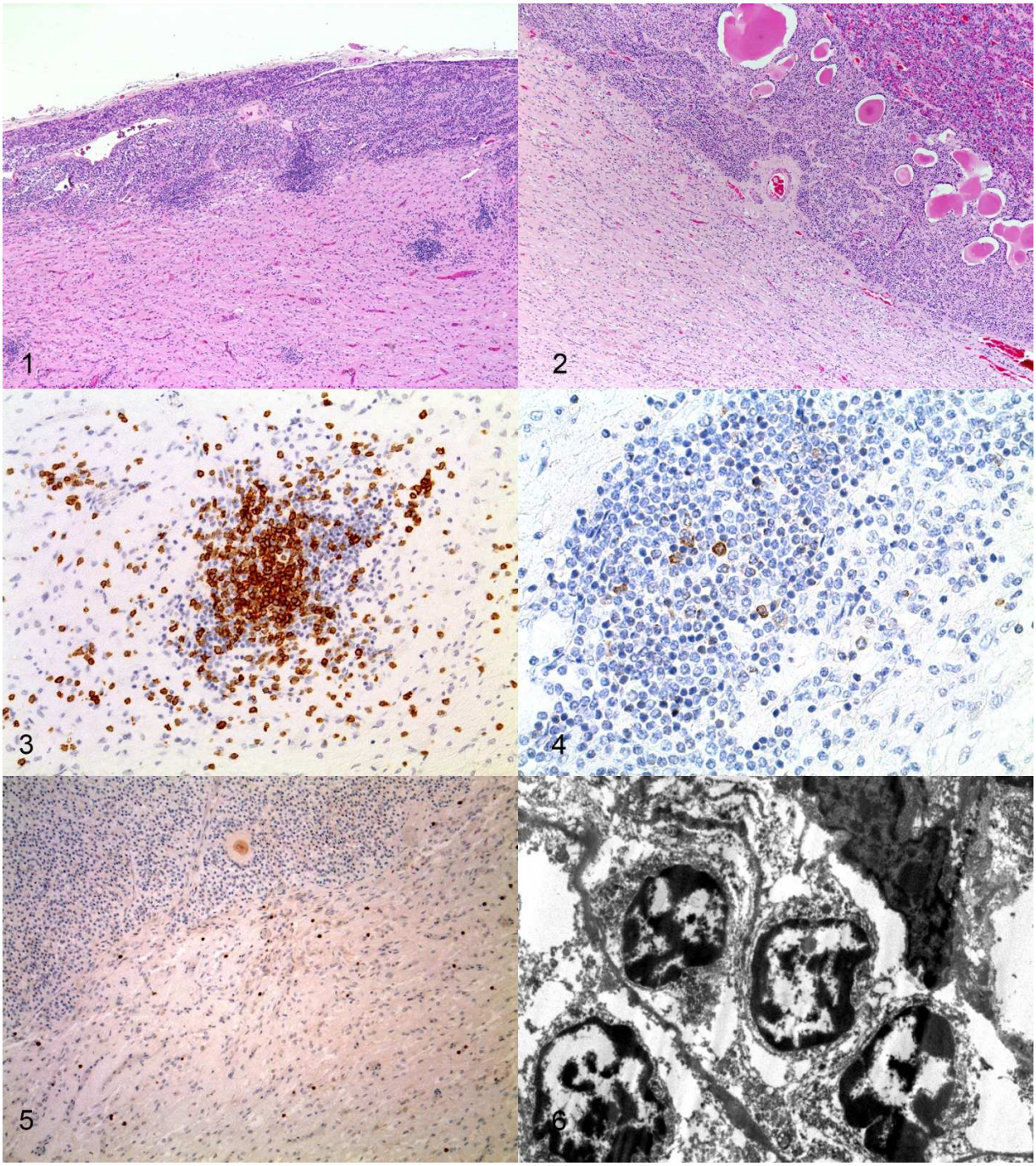


Figure 1. Pituitary gland with lymphocytic hypophysitis (group 1), horse 1. Moderate multifocal lymphocytic aggregates are present within the pars nervosa and pars intermedia. H&E.

Figure 2. Pituitary gland without lymphocytes visible on H&E (group 3), horse 24. Moderate numbers of dilated colloid-filled follicles are present within the pars intermedia. H&E.

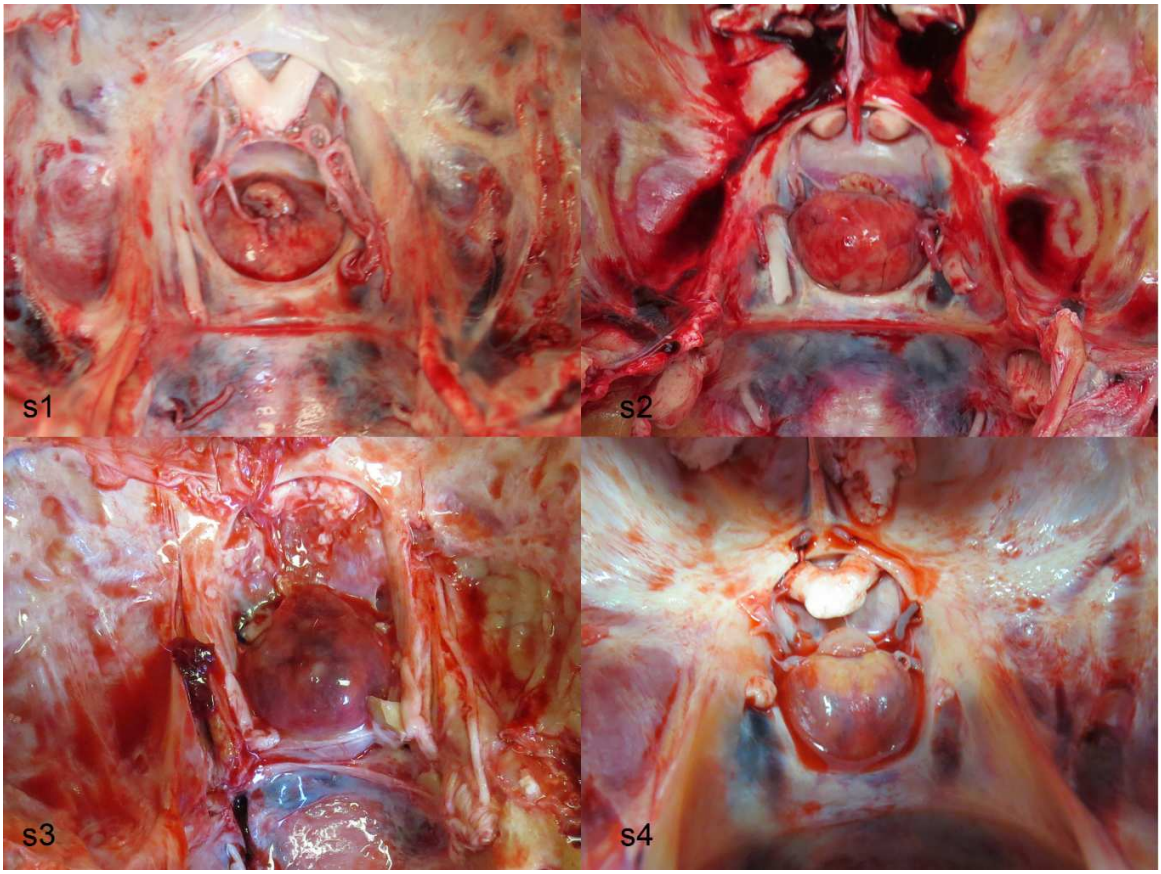
Figure 3. Pituitary gland with lymphocytic hypophysitis (group 1), horse 1. The majority of the lymphocytes show intense cytoplasmic labeling. IHC for CD3.

Figure 4. Pituitary gland with lymphocytic hypophysitis (group 1), horse 1. Low numbers of lymphocytes show cytoplasmic labelling. IHC for CD79.

Figure 5. Pituitary gland without lymphocytes visible on H&E (group 3), horse 13. Low numbers of individual, scattered lymphocytes with cytoplasmic labeling are present within pars nervosa and pars intermedia. IHC for CD3.

Figure 6. Pituitary gland with lymphocytic hypophysitis, horse 1. Four lymphocytes are seen adjacent to an endothelial cell. Transmission electron micrograph.

Supplemental figure legends



Supplemental Figure 1. Pituitary gland with lymphocytic hypophysitis, horse 1. The pituitary gland is moderately reduced in size, leaving a partially empty sella turcica.

Supplemental Figure 2. Pituitary gland with mild microscopic lymphocytic infiltrates, horse 3. The gland is moderately swollen, protruding dorsally from the sella turcica.

Supplemental Figure 3. Pituitary gland with a pituitary adenoma, horse 12. The gland is moderately swollen and has multifocal irregular red areas.

Supplemental Figure 4. Normal pituitary gland, horse 20.