



## CASE REPORT

### Quantitative Pathology of Canine Cortico-Cerebellar Degeneration

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#### ABSTRACT

The aim of this work was to document the value of morphometric analysis in quantifying neuronal loss in canine cerebellar degenerative disease. Measurements of the cerebellar cell populations in a dog affected by severe cortico-cerebellar degeneration (CCD) were compared with those of an age-matched control dog. Histological stainings (hematoxylin-eosin, thionin, Bielschowsky) were performed; the granule cell density and Purkinje cell linear density were quantified. GFAP immunohistochemistry was run to quantify the stained area. Morphometric analysis in the pathologic dog showed a significant decrease of Purkinje cell linear density (4.50/mm), granule cells density (81.34/10000  $\mu\text{m}^2$ ) and an increase of GFAP stained area (40.28%) in comparison to the age-matched dog (Purkinje cell linear density=12.74/mm, granule cell neuron density=95.53/10000  $\mu\text{m}^2$  and GFAP stained area=34.68%). The use of morphometric techniques is suggested in order to enable comparison between results obtained from different laboratories of pathology.

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#### INTRODUCTION

Cerebellar cortical degeneration (CCD) is a well-known disease related to premature aging of Purkinje cells or, more rarely, granule cells through activation of the apoptotic pathway (Nibe *et al.*, 2010). There are several reports of CCD in young and young adult dog breeds; later clinical onset is not uncommon, for example, in Gordon Setters and English Sheepdogs (Jokinen *et al.*, 2007; Gumber *et al.*, 2010; Vandeveld *et al.*, 2012). Nervous system anatomy studies used morphometry for neuronal count and distribution; in pathology, the application of this method could also be useful to quantify changes in tissues and their organization. This tool can provide a numerical, reproducible scale of quantitative features and enhance sensitivity in detecting also minimal changes (True, 1996). It could be favorably employed in the study of degenerative disorders in those cases in which some diagnostic information are lacking. Only one paper has used morphometry to study the canine cerebellum (Koyun *et al.*, 2011).

**Case history:** The dog was a male crossbreed aged 7 years and 4 months affected by slowly progressive four limbs incoordination which had started three years

previously. The last neurologic examination disclosed signs of severe cerebellar ataxia, hypermetria, spasticity and intention tremor. Brain Magnetic Resonance Imaging (MRI) showed cerebellar atrophy. Diagnosis was consistent with a cerebellar abiotrophy. The dog was euthanized due to its worsening general condition and the poor prognosis. The dog used as control was a crossbreed aged 7 years and 2 months, euthanized due to a non-neurologic disorder. The owners' consent was obtained to perform a complete necropsy of the two dogs.

**Histology and immunohistochemistry:** The dogs' brains were formalin-fixed for one week and then weighed (g). The cerebellum was weighed (g) and volume measured by liquid displacement ( $\text{cm}^3$ ) and cut according to the anatomic-functional regions (lateral and medial hemispheres, vermis) (Ghez and Thach, 2000). Each part was weighed. Hematoxylin-eosin, thionin and Bielschowsky were performed.

For immunohistochemistry sections were incubated with polyclonal anti-GFAP primary antibody (Dako, USA) overnight at +4°C. The revealing system was the streptavidin-biotin-peroxidase LSAB© kit (Dako, USA), followed by visualization with chromogen 3,3'-diaminobenzidine (DAB) (Sigma, USA).

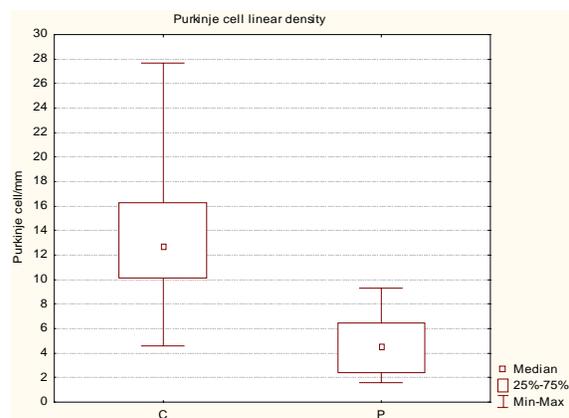
**Morphometry:** It was carried out with Image J ver 1.46 (<http://rsbweb.nih.gov/ij/index.html>). Molecular and granular cell layers thickness were manually measured (50 measurements/layer) on 11 random selected fields at 100x magnification (field area  $986315 \mu\text{m}^2$ ). The granule cell neuron density was counted on HE-stained sections. Ten randomly selected fields were acquired at 400x magnification (field area  $60400 \mu\text{m}^2$ ); automatic count of nuclei was made by the "Analyze particles" instruction after thresholding. Density was expressed as granule cell neurons/ $10000 \mu\text{m}^2$ . On thionin-sections the linear density of the Purkinje neurons was quantified on ten randomly selected fields at 200x magnification (field area  $237670 \mu\text{m}^2$ ) for each anatomic-functional region. Purkinje neurons were counted (neurons/millimeters). On GFAP-stained sections the percentage area occupied by DAB was measured by color segmentation on ten randomly selected fields at 400x magnification in the molecular layer. Statistical analysis was performed with the Statistica 8<sup>TM</sup> software. Data were tested for distribution with the Shapiro-Wilk test. For normally distributed data Student T-test was used while for not normally distributed data the Mann-Whitney U test or the Kruskal-Wallis test was used. Significance was considered for  $P < 0.05$ .

## RESULTS

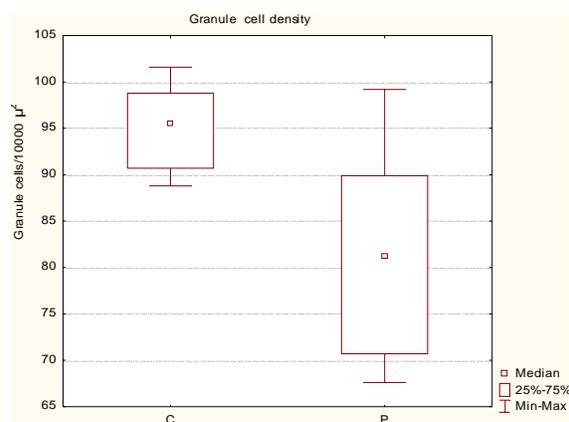
At necropsy the cerebellum of the pathologic dog was reduced in size, exposing the caudal fossa. The cerebellar folia were thinner than in the control and a widening of cerebellar sulci was evident. No macroscopic changes were present in the other organs examined. The formalin-fixed cerebellum weighed 4.14g and had a volume of  $4 \text{ cm}^3$ , the cerebellum/brain ratio weight was 6.2%; the formalin-fixed cerebellum of the control dog weighed 8.14g and had a volume of  $9 \text{ cm}^3$ ; the cerebellum/brain ratio weight was 9.6%. In comparison with the control dog (Fig. 4a) the HE-stained cerebellum of the pathologic dog showed a severe and diffuse loss of Purkinje cells (Fig. 4b); with respect to healthy neurons (Fig. 4a, inset), remaining neurons of the pathologic case displayed severe degeneration (chromatolysis, necrosis) (Fig. 4b, inset). In comparison with the control dog (Fig. 4c) the Bielschowsky silver stain emphasized the processes of basket cell neurons (the so-called "empty baskets" due to Purkinje cell loss) (Fig. 4d, inset). A moderate Bergmann gliosis was detectable with respect to the control dog by GFAP immuno-staining (Fig. 4e and 4f).

Data on Purkinje cell density were not normally distributed (S-W test:  $W=0.94$ ;  $P=0.01$ ). No differences in Purkinje cell linear density were found in the three anatomical sites considered in either dog (data not shown). The linear density of Purkinje cells evaluated on thionin-stained sections was significantly lower (M-W U test:  $U=35.00$ ;  $Z=6.10$ ;  $P=0.00$ ) in the pathologic dog (median= $4.50/\text{mm}$ ; range: 1.60-9.30) than in the control dog (median= $12.74/\text{mm}$ ; range: 4.56-27.67mm) in all anatomic-functional regions (pooled data) (Fig. 1).

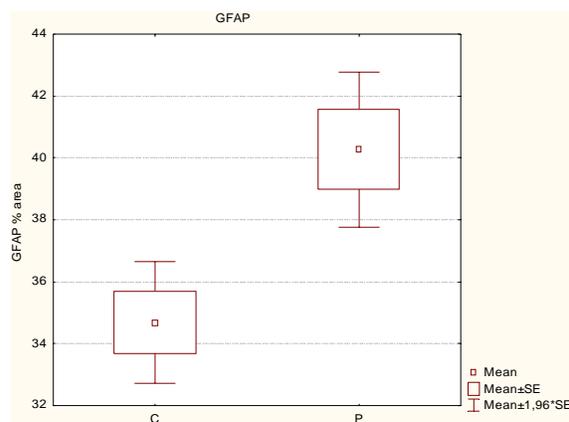
Data on molecular layer thickness were normally distributed (S-W test:  $W=0.98$ ;  $P=0.6219$ ); molecular layer thickness was significantly lower ( $t=3.192$ ;  $P=0.001897$ ) in the pathologic dog ( $256.454 \pm 59.37 \mu\text{m}$ ) in respect to control dog ( $295.722 \pm 63.560 \mu\text{m}$ ).



**Fig. 1:** The linear density of Purkinje cells of the pathologic dog (P) is significantly lower than in the control (C).

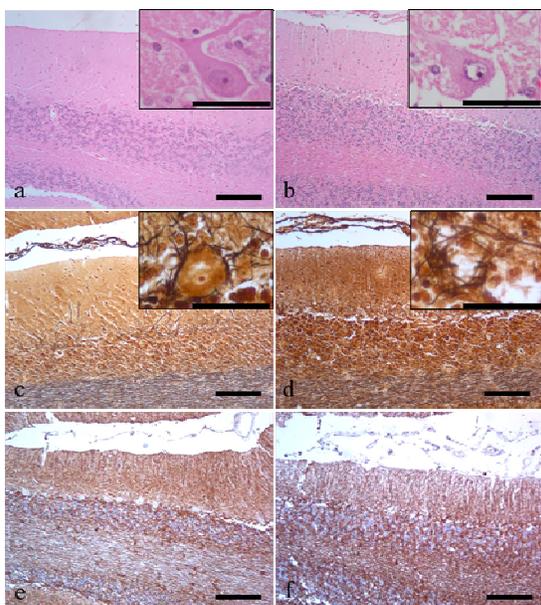


**Fig. 2:** Granule cell neuron density was significantly lower in the pathologic dog (P) than in the control (C).



**Fig. 3:** The percentage GFAP-positive area was significantly larger in the pathologic dog (P) than in the control (C).

Data on granular layer thickness were not normally distributed (S-W test:  $W=0.969$ ,  $P=0.01941$ ); granular layer thickness was not significantly different between animals (M-W U test  $1052.00$ ,  $Z=1.364$ ,  $P=0.172$ ). In pathologic dog granular cell layer median thickness was  $171.174 \mu\text{m}$  ( $96.453-269.924 \mu\text{m}$ ), while in control dog granular layer median was  $192.717 \mu\text{m}$  ( $68.772-363.938 \mu\text{m}$ ). Data on granule cell neuron density were not normally distributed (S-W test:  $W=0.88$ ;  $P=0.01717$ ).



**Fig. 4:** a,b Cerebellum, hematoxylin-eosin stain (bar=200µm). Control dog (a) compared to pathologic dog (b) showing a severe and diffuse loss of Purkinje cells and a severe degeneration of the remaining neurons (insets bar=50µm). c,d Cerebellum, Bielschowsky silver stain (bar=200µm). Control dog showing basket cells around Purkinje cell (c) compared to pathologic dog (d) showing the so-called “empty basket” (insets bar=50µm). e,f Cerebellum, GFAP immuno-staining (bar=200µm). With the respect to control dog (e) a diffuse reactive Bergmann gliosis is present.

Granule cell neuron density, measured only in the vermis, was significantly lower (M-W U test:  $U=18.00$ ;  $Z=2.61$ ;  $P=0.009175$ ) in the pathologic (median =  $81.34/10000\mu\text{m}^2$ ; range: 67.64-99.24) than in the control dog (median =  $95.53/10000\mu\text{m}^2$ ; range: 88.80-101.58) (Fig.2).

Data on GFAP labelling were normally distributed (S-W test:  $W=0.92$ ;  $P=0.1151$ ). The percentage area occupied by GFAP-labelled cells was significantly higher ( $t=-3.50$ ;  $P=0.002537$ ) in the pathologic ( $40.28\pm 3.83\%$ ) than in the control dog ( $34.68\pm 3.31\%$ ) (Fig. 3).

## DISCUSSION

CCD usually presents as a progressive postnatal disorder and is classically described among the degenerative diseases of the central nervous system. CCD is due to a premature aging of Purkinje cells; microscopically it is characterized by a loss of Purkinje neurons accompanied by a consequential reduction of granule cell neurons (Summers *et al.*, 1995). In this study morphometry proved a useful ancillary tool to measure the residual cerebellar populations. In particular, it showed that the Purkinje cell linear density was significantly lower in the CCD dog compared to the control dog in each anatomic-functional region. Typically, the involvement of the cerebellum in CCD is not uniform, beginning first in the vermis and paramedian lobule and spreading to the lateral hemispheres (Summers *et al.*, 1995; Vandavelde *et al.*, 2012). In this case the loss of Purkinje cells was total and involved the entire cerebellum, reinforcing the longstanding nature of the process. CCD is frequently associated with a

reactive gliosis (Summers *et al.*, 1995; Vandavelde *et al.*, 2012); GFAP was highly expressed in the pathologic case described here, as in other dogs with CCD reported in literature (Nibe *et al.*, 2010).

Moreover in this case, the thickness of the molecular layer follows a normal distribution, while the thickness of the granular cell layer is not normal: these two statistical trends reflect the distribution pattern of GFAP stained area (normal distributed) and the granule cell density (not normal distributed). In fact, it is known that Purkinje cell death is patterned and topographically complex (Sarna and Hawkes, 2003); as a consequence in this pathologic case Purkinje linear density was not normally distributed and the granule cell layer was depleted consistently in a patterned way, following the same topography and statistical distribution. Indeed, patterned death was attended by a diffuse reactive Bergmann astroglia, probably aiming to replace shrunken and depleted Purkinje branchlets and dendritic arborisations.

The employment of morphometry in the study of degenerative disorders could provide an interesting contribution to cases with more subtle histological changes or to those in which some diagnostic information (i.e. MRI data or the weight of cerebellum) is lacking.

**Conclusion:** This paper has presented the morphometric parameters of the cerebellum of a dog with CCD in comparison with those of an age-matched control; these data are not present in the literature and need to be compared with additional cases. The use of morphometric technique is also suggested in order to compare the measurements obtained from different laboratories of pathology.

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