Analysis of blood degradation products and ferritin in the cerebrospinal fluid of dogs with acute thoracolumbar intervertebral disk extrusion, a prospective pilot study

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Abstract

Background: Hemorrhage in the spinal canal leads to further damage of the spinal cord influencing outcome in dogs with intervertebral disk (IVD) extrusion. The aim of the study was to evaluate blood degradation products and ferritin in the cerebrospinal fluid (CSF) of dogs with thoracolumbar IVD extrusion, and their association to clinical parameters and MRI findings.

Results: In the CSF of dogs with IVD extrusion, both net oxyhemoglobin absorption (NOA) and net bilirubin absorption (NBA) were significantly higher compared to the control groups of dogs with steroid responsive meningitis arteritis (SRMA) and idiopathic epilepsy (IE) (P < 0.001), but NOA compared to the idiopathic epilepsy group contaminated artificially with blood (IEc) was not (P = 0.890). Ferritin concentration was significantly higher in dogs with IVD extrusion compared to dogs with IE (P = 0.034), but not to dogs with SRMA (P = 0.526). There was no association between NOA, NBA or ferritin concentration and severity or duration of clinical signs. In dogs with a higher ferritin concentration the outcome was better (P = 0.018). In dogs with evidence of hemorrhage on MRI, NOA and NBA were significantly higher (P = 0.016, P = 0.009), but not ferritin (P = 0.0628).

Conclusion and clinical importance: Quantification of blood degradation products and ferritin in the CSF of dogs to assess subarachnoidal hemorrhage is feasible; however, larger case numbers are needed to evaluate the relevance of NBA and ferritin as prognostic indicators.

Keywords: Spinal cord injury, Oxyhemoglobin, Bilirubin, Subarachnoidal hemorrhage

Background

In paraplegic dogs with thoracolumbar intervertebral disk (IVD) extrusion, the presence or absence of nociception is considered the most important indicator to determine the prognosis [1, 2], and generally correlates with histopathological findings [3]. However, the outcome of surgically treated paraplegic dogs with absent nociception varies widely [2, 4–6]. is highlights the fact that other factors beyond this contribute to recovery or lack thereof. Several other prognostic factors were previously studied including severity and duration of clinical signs, site of disk extrusion, specific magnetic resonance imaging (MRI) findings [7–9], and cerebrospinal fluid (CSF) characteristics [10–17].

After the primary mechanical insult on the spinal cord in IVD extrusion, secondary mechanisms of injury including decreased vascular perfusion followed by ischaemia and perivascular edema, electrolyte imbalances, glutamatergic excitotoxicity, oxidative stress, inflammation, and apoptosis lead to further damage of the spinal cord parenchyma [18, 19]. Additionally, there is strong evidence that intramedullary and subdural hemorrhage...
has an important impact on the development of secondary injury and outcome [20, 21]. Therefore, monitoring hemorrhage in the subarachnoidal space would seem useful to assess spinal cord injury (SCI) and hence prognosis.

Following hemorrhage into the subarachnoidal space, rapid lysis of red blood cells occurs, thereby releasing oxyhemoglobin. The liberated oxyhemoglobin is gradually converted into bilirubin and free iron by macrophages and other cells of the leptomeninges [22]. The free iron is detoxified by ferritin [23, 24].

Detection of such products in CSF with spectrophotometry for net oxyhemoglobin absorption (NOA) and net bilirubin absorption (NBA) as well as detection of ferritin in the CSF is used in people with subarachnoidal hemorrhage (SAH) when computed tomography of the head is negative for hemorrhage [24, 27, 28]. Spectrophotometry for blood degradation products in the CSF of animals has been used experimentally in a model for human SAH [28, 29], but to our knowledge, never before to detect blood degradation products in the CSF in a naturally occurring disease. Neither have these techniques been used before to assess SCI.

The aim of the present pilot study was to investigate the utility of spectrophotometry and ferritin ELISA to quantify blood degradation products and ferritin in CSF of dogs with SCI following IVD extrusion. Further, we wanted to assess whether these degradation products and ferritin are correlated with neurological grade of affected dogs and their outcome, and whether they can be used as prognostic indicators. We hypothesized that quantification of blood degradation products and ferritin in the CSF could be a method to assess subdural hemorrhage and may serve as useful prognostic indicator because of its influence on secondary damage of the spinal cord [3, 20, 21, 25, 26]. Additionally, the correlation of blood degradation products and ferritin with MRI findings was evaluated.

Results

Clinical data

The study population consisted of 58 purebred dogs (33 different breeds), and 11 mixed-bred dogs. Pure breeds represented by more than three dogs were dachshunds ($n = 10$) and French bulldogs ($n = 7$). Forty-eight dogs were male (21 castrated), and 21 were female (13 spayed). The mean age of the dogs was 4.8 years (range, 6 months to 12.8 years). The median body weight of the dogs was 13.5 kg (range, 3.4 to 81.0 kg).

In dogs with IVD extrusion, the initial neurological condition was Grade I in 1 dog, Grade II in 12, Grade III in 9, Grade IV in 14, and Grade V in 3 dogs.

The median duration of the clinical signs until CSF tap was 2 days (range, 1 to 21 days), and the clinical signs were classified as acute in 20 dogs, subacute in 13 dogs, and chronic in 6 dogs, accordingly. Three of the chronically affected dogs displayed a worsening of clinical signs within the last 24 h.

The IVD extrusion site was at T11-T12 in three dogs, T12-T13 in 13 dogs, T13-L1 in 12 dogs, L1-L2 in one dog, L2-L3 in four dogs, L3-L4 in two dogs, L4-L5 in three dogs, and L5-L6 in one dog. Time of discharge or euthanasia was between 0 and 17 days (median 4). Thirty dogs had a good outcome and were ambulatory, one dog showed an improvement, but was not ambulatory at time of discharge. The remaining 8 dogs were euthanized immediately after imaging ($n = 2$ of each; neurological grade IV and V), after deterioration of neurological signs ($n = 3$; neurological grade II to III, grade II to V, and grade IV to V), and because of lack of improvement ($n = 1$, neurological grade IV).

Magnetic resonance imaging

On MRI, hemorrhage was evident in 24 dogs, whereas in the 15 remaining dogs no signs of hemorrhage were visible.

Serum bilirubin levels and cerebrospinal fluid analysis

Median values and ranges of serum bilirubin, CSF total protein, WBC count, NBA, NOA, and ferritin concentration, results of pandy testing and CSF cytology, and the classification according to Cruickshank et al. of the different groups are given in Table 1.

Spectrophotometric analysis of NOA and NBA has been performed in 33 of 39 dogs with IVD extrusion, and routine CSF analysis in 6 of these 33 dogs because of insufficient quantity of CSF.

Statistical analysis

Comparing the IE and IEc group, NOA was significantly higher in the IEc group ($P < 0.001$), and NBA and ferritin concentration were not different between both groups ($P = 0.272$, $P = 0.546$, respectively). (Fig. 1a-c).

The NOA and NBA were significantly higher in the CSF of dogs with IVD extrusion compared to dogs with IE ($P < 0.001$) and SRMA ($P < 0.001$). (Fig. 2a, b).

The CSF ferritin concentration was significantly higher in dogs with IVD extrusion compared to dogs with IE ($P = 0.034$), but not to dogs with SRMA ($P = 0.526$). (Fig. 2c).

Using the classification according to Cruickshank et al. [27] (Fig. 4), dogs with IVD extrusion showed significantly more often hemorrhage in the subarachnoidal space than dogs with IE and SRMA (both $P < 0.001$). Comparing the non-contaminated and contaminated CSF of the IE dogs according to this classification, the IEc showed significantly more often hemorrhage in the subarachnoidal space than the IE ($P = 0.002$).
In dogs with IVD extrusion, there was no association be-
tween NOA, NBA, or ferritin concentration and the initial
neurological grade (\(P = 0.104\), \(P = 0.081\), \(P = 0.704\), re-
spectively), and duration of clinical signs (\(P = 0.411\), \(P = 0.218\), \(P
= 0.663\), respectively). There was also no association be-
tween NOA or NBA and the outcome (\(P = 0.967\), \(P = 0.310\), re-
spectively), but dogs with a higher ferritin concentration
in the CSF had a better outcome (\(P = 0.018\)).

Using the classification according to Cruickshank et al.
there was no association to the outcome (\(P = 0.733\)).

In dogs with evidence of hemorrhage on MRI, there was
a significantly higher NOA and NBA (\(P = 0.016\), \(P = 0.009\), re-
spectively), but no association with ferritin (\(P = 0.628\)).

**Discussion**

We have shown before that hemorrhage in SCI is associ-
ated with the severity of white and gray matter damage
and the longitudinal extension of myelomalacia [21]. In
addition to the mechanical effects of hemorrhagic cord
necrosis [21], it has been a long held view that disruption
of the blood-spinal cord barrier with hemorrhage follow-
ing SCI leads to exposure of the myelon to destructive ef-
facts of, among others, cytotoxic neurotransmitters [35],
cytokines [36], vasoactive peptides [37], oxygen free radi-
cals [38], endothelin-1 [26, 39, 40] and heme-oxygenase-1
[41]. We hypothesized that detection of blood degradation
products in the CSF could be a promising prognostic indi-
cator [21]. A major challenge is to distinguish intra vitam
from iatrogenic hemorrhage resulting from CSF puncture.
Indeed as reported in people and dogs previously [42, 43],
the latter was often suspected since we used lumbar punc-
ture in view of the scope of this study. Counting erythro-
cytes or measuring hemoglobin cannot be used to
distinguish between iatrogenic and true hemorrhage [44].

**Table 1 Median values and ranges of serum bilirubin levels, cerebrospinal fluid analysis, spectrophotometric and ferritin measurements**

|                      | Intervertebral disc extrusion (\(n = 39\)) | Idiopathic epilepsy (\(n = 21\)) | Idiopathic epilepsy contaminated (\(n = 21\)) | Steroid-responsive meningitis-arteritis (\(n = 9\)) |
|----------------------|-------------------------------------------|---------------------------------|---------------------------------------------|-----------------------------------------------|
| **Routine CSF analysis** |                                           |                                 |                                             |                                               |
| WBC / μl             | *                                         |                                 |                                             |                                               |
| median (range)       | 8 (1–14)                                  | 0.67 (0–5)                      |                                             | 47.67 (66–1680)                               |
| TP (g/l)             | *                                         |                                 |                                             |                                               |
| median (range)       | 0.73 (0.14–8.55)                          | 0.12 (0.11–0.23)                | 0.25 (0.15–0.8)                             |                                               |
| Pandy (number)       | *                                         |                                 |                                             |                                               |
| –                    | 2                                         | 17                              | 5                                           |                                               |
| +                    | 3                                         | 4                               | 4                                           |                                               |
| ++                   |                                            |                                 |                                             |                                               |
| +++                  |                                            |                                 |                                             |                                               |
| **Cytology (number)**|                                           |                                 |                                             |                                               |
| mononuclear          | 2                                         |                                 |                                             |                                               |
| neutrophilic         | 1                                         |                                 |                                             |                                               |
| **CSF spectrophotometry** |                                         |                                 |                                             |                                               |
| NOA (AU)             | **                                        |                                 |                                             |                                               |
| median (range)       | 0.073 (0–3.585)                           | 0.002 (0–0.009)                 | 0.064 (0.003–1.505)                         | 0.004 (0–0.010)                               |
| NBA (AU)             | **                                        |                                 |                                             |                                               |
| median (range)       | 0.016 (0–0.373)                           | 0.001 (0–0.007)                 | 0.001 (0–0.011)                             | 0.002 (0.001–0.008)                           |
| **ELISA CSF Ferritin (ng/ml)** |                                         |                                 |                                             |                                               |
| median (range)       | 41.28 (15.08–228.19)                      | 33.90 (13.58–64.00)             | 36.73 (15.00–653.30)                        | 31.11 (17.24–125.14)                         |
| **Serum Bilirubin (μmol/l)** |                                         |                                 |                                             |                                               |
| median (range)       | 2 (0.7–6)                                 | 2 (0.7–6)                       | 2 (1–4.2)                                  |                                               |
| **Classification of the result according to Cruickshank [27]** |                                           |                                 |                                             |                                               |
| Subarachnoidal hemorrage: |                                         |                                 |                                             |                                               |
| yes                  | 26                                        | 0                               | 1                                           | 1                                             |
| no                   | 6                                         | 21                              | 13                                          | 8                                             |
| not excluded         | 1                                         | 0                               | 7                                           | 0                                             |

*performed in 6 dogs, **performed in 33 dogs, WBC, white blood cell count; TP, total protein; AU, absorption units; NOA, net oxyhemoglobin absorption; NBA net bilirubin absorption; CSF, cerebrospinal fluid
Fig. 1 Comparison of (a) net oxyhemoglobin absorbance (NOA), (b) net bilirubin absorbance (NBA), and (c) ferritin concentration in the cerebrospinal fluid (CSF) of dogs with idiopathic epilepsy either non-contaminated (IE) or artificially blood contaminated (IEc). The box represents the 25th, 50th, and 75th percentile of the distribution; the whiskers approximate the 5th–95th percentile; * indicates values of \( P < 0.05 \).

Fig. 2 Comparison of (a) net oxyhemoglobin absorbance (NOA), (b) net bilirubin absorbance (NBA), and (c) ferritin concentration in the cerebrospinal fluid (CSF) of dogs with intervertebral disc extrusion (IVDE) to dogs with idiopathic epilepsy (IE) and dogs with steroid responsive meningitis arteritis (SRMA). The box represents the 25th, 50th, and 75th percentile of the distribution; the whiskers approximate the 5th–95th percentile; * indicates values of \( P < 0.05 \).
Detection of erythrophages and siderophages in the CSF can provide information about the age and dynamics of the bleeding, however, studies in the human literature included too few patients to estimate sensitivity for SAH [44, 45]. Spectrophotometry of CSF for bilirubin and oxyhemoglobin is recommended by the British national guidelines in human patients with suspected SAH when computed tomography is negative in order to determine the need for angiography [27]. Spectrophotometry has an estimated sensitivity of 87–100% and specificity of 75–99% for aneurysmal SAH in human patients [31, 46–48]. Bilirubin in CSF may serve as an indicator of central nervous system hemorrhage but only in the absence of severe blood-CSF barrier breakdown, and jaundice [27, 42]. Hence, a decision tree which takes the NOA, NBA, serum bilirubin and CSF protein concentration into consideration is recommended in the British national guidelines to interpret spectrophotometric results from human patients [27]. In the present study, we found that the same decision tree could be used for the assessment of spinal cord hemorrhage in dogs, with the limitation of traumatic puncture.

Following hemorrhage into the subarachnoidal space, rapid lysis of red blood cells occurs within two to four hours as a consequence of the low CSF osmolality leading to release of oxyhemoglobin, which may be detected between two and 12 h up to one week after the onset of bleeding [44]. However, oxyhemoglobin can also result from in vitro hemolysis after a traumatic puncture [43].

Accordingly, in the present study, NOA was significantly higher following artificial blood contamination of the CSF and subsequent centrifugation within 15 min compared to the same CSF without contamination. Therefore, NOA has a relatively high probability of falsely elevated results especially in lumbar CSF taps due to traumatic puncture in the face of iatrogenic blood contamination, and thus rendering the interpretation of significantly elevated NOA in dogs with IVD extrusion difficult. We concluded that NOA may be of limited value to assess cord hemorrhage.

The liberated oxyhemoglobin is gradually converted into bilirubin and free iron by macrophages and other cells of the leptomeninges [22]. Thus, an elevation of bilirubin cannot be explained by a traumatic puncture, as reflected by the NBA results clearly distinguishing between artificially blood contaminated and non-contaminated CSF of IE dogs. The conversion into bilirubin and iron is detectable about 12 h after onset of hemorrhage [27, 29], increases over approximately one week, and remains detectable for two to four weeks after the hemorrhage in people [22, 31]. In the present study, all CSF taps were performed after more than 12 h and less than 3 weeks after the beginning of clinical symptoms, and we could show a significant elevation of NBA in dogs with SCI compared to controls. However, we could not find a correlation between NBA values and initial neurological grade, duration of clinical signs and outcome in dogs with IVD extrusion which may be due to several variables. The level of bilirubin in the CSF is not only dependent on the quantity of hemorrhage, but also very much on the time point of CSF puncture after SCI, and hence the conversion stage of oxyhemoglobin. The interpretation of NBA values may be further complicated by progression of the IVD disease. For example, in the present study, three of six chronically affected dogs showed an acute worsening of clinical signs possibly by further extrusion of IVD material [49–51] perhaps leading to new hemorrhage. Beside the stage of hemorrhage, the distance of the primary lesion site to the site of CSF tap could also have an influence on the results. In the present study, the CSF tap was performed at L5–6 or L6–7 and the distance to the IVD extrusion site was between zero and seven vertebral bodies.

Ferritin is the main extra-cellular iron transporter, and located in every organ. After hemorrhagic events, it is synthesized by several cell types among others macrophages in order to bind free iron, and to prevent the occurrence of free radicals that damage tissues [52]. The free iron gets detoxified by ferritin which reaches a maximum peak after about eight to 11 days following hemorrhage [23, 24]. The ferritin concentration was significantly higher in the CSF of dogs with IVD extrusion compared to dogs with IE, but not compared to dogs with SRMA. The latter is consistent with previous observations according to which ferritin concentration in the CSF is also increased in inflammation of the central nervous system, especially in pyogenic inflammation [52].

Interestingly, dogs who recovered ambulation had a significantly higher ferritin concentration than dogs with a poor outcome. However, in addition to hemorrhage as a source of iron, the ferritin concentration could also reflect the inflammatory response following SCI, which is extremely complex and has both beneficial and deleterious effect [53–56]. In the spinal cord, ferritin is a marker of microglia, which is important for reestablishing tissue homeostasis after SCI [55, 57–59]. However, the interpretation of ferritin levels in canine CSF was also complicated by its wide concentration range in the control dogs. It normally does not cross the blood brain barrier due to its relative high molecular weight of 450 kD [60], and no correlation between CSF and serum ferritin concentration has been found in people. The upper normal range of ferritin in human CSF is 12 ng/ml. The level of ferritin in the CSF of control dogs with IE in the present study was between 13 and 64 ng/ml. However, to evaluate the normal range of ferritin in the CSF of dogs, a larger case number of completely healthy dogs should be examined.

MRI is the method of choice to evaluate the spinal canal for the presence of hemorrhage until now. However, to
distinguish between extradural and subdural hemorrhage, and especially its quantification remains difficult. The amount of blood degradation products in the CSF correlated with the evidence of hemorrhage on MRI in the present study.

Limitations of the present pilot study are the very low number of paraplegic dogs with loss of nociception and the short follow up time of dogs with a poor outcome probably leading to biased statistical results. Additionally the variation in clinical duration (acute vs. chronic) may have affected the measurements. Larger case numbers are needed. Also intraparenchymal hemorrhage is most likely missed with measurements of blood degradation products in the CSF.

Conclusion
The results of this study show that detection of blood degradation products such as oxyhemoglobin, bilirubin, and ferritin in lumbar CSF to demonstrate hemorrhage in the subarachnoidal space of dogs following IVD extrusion is feasible, and correlates with MRI findings. However, while significant differences were found between dogs with IVD disease and controls, correlations of NOA and NBA to the initial neurological grade and the outcome in individual animals could not be found. NOA is not recommended after a lumbar CSF puncture due to the high risk of iatrogenic blood contamination. Paradoxically, dogs who regained ambulation had significantly higher CSF ferritin concentration.

Since the generation of blood degradation products in the CSF is a highly dynamic process bilirubin and ferritin values depend very strongly on the duration of clinical signs. Larger numbers of animals for each defined point in time following initial SCI are necessary to evaluate the usefulness of bilirubin and ferritin as prognostic indicators.

Methods
Case selection
Examination of the CSF for blood degradation products was performed prospectively in 39 dogs with thoracolumbar IVD extrusion which were presented between September 2013 and October 2015 at the veterinary teaching hospital of the University of Bern. Inclusion criteria were diagnosis of surgical or histopathologically confirmed thoracolumbar IVD extrusion, well-documented clinical records (breed, age, gender, duration of clinical signs, neurological grade, MRI findings, outcome), and sufficient quantity of CSF (0.5 ml) for measurements. Additionally, CSF of 21 dogs with idiopathic epilepsy (IE) and 9 dogs with steroid responsive meningitis arteritis (SRMA) served as controls.

Clinical data
Breed, age and gender of all dogs were recorded, and a complete neurological examination was performed at the time of presentation. In dogs with thoracolumbar IVD extrusion, duration and severity of clinical signs, site of IVD extrusion, and outcome were noted.

The initial neurological condition was graded on a 1 to V scale as published before [3]: Grade I, spinal hyperesthesia only; Grade II, ambulatory paraparesis, ataxia, and proprioceptive deficits; Grade III, non-ambulatory paraparesis; Grade IV, paraplegia with present nociception; and Grade V, paraplegia with loss of nociception. In case of grade differences between left and right limbs, the more severe grade was assigned.

The neurological condition was reevaluated at time of discharge. The outcome was determined as follows: grade 0 - lack of improvement or euthanasia; grade 1 - improvement of the neurological status by at least one grade, but not able to walk without support; grade 2 - recovery to ambulation [20].

In all included dogs with thoracolumbar IVD extrusion, the diagnosis was provided by MRI and confirmed during standard hemilaminectomy.

The neurological condition was reevaluated at time of discharge. The outcome was determined as follows: grade 0 - lack of improvement or euthanasia; grade 1 - improvement of the neurological status by at least one grade, but not able to walk without support; grade 2 - recovery to ambulation [20].

In control dogs, the diagnosis of IE or SRMA was based on signalement, history, clinical signs and specific diagnostic testing. Dogs with IE displayed no interictal neurological deficits, metabolic causes were excluded, and MRI of the head and CSF tap were unremarkable. Dogs with SRMA revealed suspicious clinical signs (neck pain, pyrexia), a CSF tap with neutrophilic pleocytosis and without detectable pathologic organisms on cytology, an increased immunoglobulin A level in serum and CSF, and a resolution of clinical signs after treatment with corticosteroids.

Magnetic resonance imaging
MRI examinations were performed in all dogs using a 1.0-T magnet. The MRI protocol usually included a transverse and sagittal T2-weighted fast spin-echo sequence, a sagittal fluid-attenuating inversion recovery sequence (FLAIR), a dorsal short tau inversion recovery sequence (STIR), dorsal and transverse T1-weighted sequences, and a transverse T2*-weighted sequence. Board-certified radiologists qualitatively evaluated the MRI images and judged hemorrhage in the spinal canal (epidural, subdural) as present or not present.

Serum bilirubin levels and cerebrospinal fluid analysis
A blood sample of all dogs was taken by venipuncture at the time of CSF collection, and serum bilirubin levels were determined [31].
For the measurements of blood degradation products and ferritin, a minimum of 0.5 ml CSF was needed. The CSF tap was performed under general anesthesia at the cisterna magna in dogs with SRMA, and subsequently following MRI in dogs with IE. A lumbar tap (L5–6 or L6–7) was performed following MRI in dogs with thoracolumbar IVD extrusion.

In dogs with IVD extrusion, 0.5 ml of the CSF was processed immediately for measurements of blood degradation products (see below), and if provided that a sufficient quantity of CSF remained, routine CSF analysis was performed including white blood cell (WBC) count, total protein, pandy testing, and in cases with a WBC count > 5 / microliter cytology following cytospin preparation.

To evaluate the potential effect of blood contamination during CSF puncture on spectrophotometric and ferritin measurements, the CSF samples from the 21 dogs with IE were divided in two parts (each at least 0.5 ml), and one part was artificially contaminated with a drop of blood (IEc).

All CSF samples, were protected from light immediately after collection, centrifuged (10 min, 2360 g, 4 °C) within 15 min, and the supernatant stored in Eppendorf-tubes at −20 °C until further diagnostics [27, 32, 33]. The samples were analyzed at wave lengths between 350 and 660 nm using a Shimadzu UV-1800 spectrophotometer in the Center of Laboratory Medicine, University Institute of Clinical Chemistry (Inselspital). NOA and NBA were determined in the CSF according the Chalmers’ calculation [34]. A predicted baseline, which forms a tangent to the scan between 350 and 400 nm and again between 430 and 530 nm was drawn. The absorbance above this predicted baseline at 476 nm reflects the NBA. If the baseline forms a tangent to the scan before 476 nm, the measured NBA is by definition zero. The NOA is represented by any oxyhemoglobin peak above this predicted baseline (Fig. 3) [27].

Additionally, the measurements were interpreted in respect to the serum bilirubin and CSF total protein level by following a recommended decision tree [27]: (1) no evidence of SAH, or (2) consistent with SAH, or (3) SAH cannot be excluded (Fig. 4).

Ferritin was determined quantitatively in the CSF using a commercially available canine ferritin ELISA kit. Every sample was measured in duplicates. Subsequently, the mean was calculated and used for statistical evaluation.

**Statistical analysis**

Dogs were assigned to one of the following four groups: (1) dogs with thoracolumbar IVD extrusion, (2) dogs with IE, (3) dogs with IE and CSF artificially blood contaminated (IEc), and (4) dogs with SRMA.

For statistical analysis, the neurological grade of dogs with IVD extrusion at the initial presentation was classed together to ambulatory paraparesis (grade I, II), non-ambulatory paraparesis (grade III), and paraplegia (grade IV, V). Due to small case numbers, the outcome grades 0 and 1 were merged.

Statistical evaluation was performed using the software package NCSS 9 (http://www.ncss.com). The threshold value for statistical significance was set at P < 0.05.

Since data were not normally distributed non-parametric tests were used. The Kruskal-Wallis test was used to evaluate the equality of variance for NOA, NBA, ferritin, and the classification according to Cruickshank et al. [27]. Multiple comparisons between dogs with IVD extrusion and the control dogs with IE or SRMA were performed using the Kruskal-Wallis test and the Wilcoxon two-sample test, respectively. The influence of iatrogenic blood contamination was evaluated by comparing NOA, NBA, ferritin concentration, and the classification according to Cruickshank et al. [27] between the IE and IEc group using the Wilcoxon two-sample test.

**Fig. 3** Representative spectrophotometric scan (blue curve). A predicted baseline (black), which forms a tangent to the scan between 350 and 400 nm and between 430 and 530 nm was drawn. The vertical reference line at 476 nm represents the net bilirubin absorbance (green), and the vertical reference line at any peak between 410 and 418 nm the net oxyhemoglobin absorbance (red).
In dogs with thoracolumbar IVD extrusion, associations between NOA, NBA, ferritin, and the classification according to Cruickshank et al. [27] in the CSF and (1) the initial neurological grade, (2) the duration of clinical signs, (3) the outcome, and (4) MRI findings were statistically evaluated using the Kruskal-Wallis test and the Wilcoxon two-sample test, respectively. Results are given as median and range (5th–95th percentile).

Endnotes
1Panorama HFO, Philips Medical Systems
2UV/VIS spectrophotometer Shimadzu UV-1800, Shimadzu, Kyoto, Japan
3Canine ferritin ELISA kit, BlueGene Biotech, Putuo District, Shanghai, China

Abbreviations
CSF: Cerebrospinal fluid; IE: Idiopathic epilepsy; IEc: Idiopathic epilepsy contaminated; IVD: Intervertebral disk; MRI: Magnetic resonance imaging; NBA: Net bilirubin absorption; NOA: Net oxyhemoglobin absorption; SAH: Subarachnoidal hemorrhage; SCI: Spinal cord injury; SRMA: Steroid responsive meningitis arteritis; WBC: White blood cell count

Acknowledgements
The authors want to thank the Specialization Commission of the University of Bern, and the Burgergemeinde Bern.

Funding
The study was funded by the Specialization Commission of the University of Bern, and the Burgergemeinde Bern. Neither the design of the study, nor the collection, analysis, and interpretation of data and writing the manuscript was influenced by the Specialization Commission of the University of Bern or the Burgergemeinde Bern.

Availability of data and materials
The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
SB and DH conceived the study. SB and DH collected all the data and wrote the manuscript. EM and JM did the ferritin ELISA. CS did and analyzed the spectrophotometric measurements. All authors read and approved the final manuscript.

Ethics approval
The study protocol was approved by the Swiss Veterinary Service of the Office of Agriculture and Nature (LANAT), Canton Bern, Switzerland (approval number BE 14/12). The local ethical authorities at the Vetsuisse Faculty, University of Bern, approved the study. For the dogs with intervertebral disc disease a written consent from the owners was taken. For the dogs with idiopathic epilepsy the CSF tab was part of the routine diagnostics and only the leftover material was taken.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interest.

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Received: 3 January 2018 Accepted: 23 April 2019
Published online: 14 May 2019

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