Creatine kinase isoenzymes and macroenzymes in dogs with different neurological diseases

Running header: CK iso- and macroenzymes in dogs with CNS diseases

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Abstract

Background: Increased activity of creatine kinase (CK) isoenzymes and macroenzymes, and in particular of the brain isoenzyme (CK-BB) has been reported in dogs with central nervous system (CNS) disorders. However, no studies on the possible differences in serum activities of CK iso- or macroenzymes in different neurological diseases are available.

Objective: The aim of this study was to describe the electrophoretic distribution of CK iso- and macroenzymes in dogs with CNS disorders in order to assess whether this distribution depends on a specific neurological disease.

Methods: This study was done on sera from 45 dogs with neurological diseases (degenerative, n=7; idiopathic epilepsy or IE, n=14; inflammatory, n=16; space occupying lesions or SOL, n=8) and from 10 clinically healthy dogs. The separation of serum CK isoenzymes and macroenzymes was performed using an automated electrophoretic method already validated in dogs.

Results: Compared with healthy dogs, dogs with CNS disorders had a significantly higher total CK activity and CK-BB activity, and a significantly lower Macro-CK2 activity (P<0.001 for all these comparisons). Comparison of pathological subgroups and healthy dogs revealed significant differences (P<0.01) in dogs with IE and inflammatory disorders for total CK activity, in all the subgroups for CK-BB (P<0.01), and in dogs with IE and SOL for Macro-CK2 (P<0.01).

Conclusions: This suggests that CK-BB is released by neurons damaged by inflammatory or degenerative conditions or due to compressive effects of SOLs. However, the neurological diseases cannot be differentiated to each other by this approach, unless further studies will define appropriate diagnostic thresholds.

Keywords: CK; CK-BB; dog; electrophoresis; neurology;
Creatine kinase (CK; EC 2.7.3.2) catalyzes the reversible reaction between ADP and phosphocreatine to form creatine and ATP. In most species, tissues with high CK activity include skeletal and cardiac muscle, followed by neural tissue.\(^1\) The leakage of CK from injured tissues leads to increased CK activity in blood. Therefore, the plasma or serum CK activity may be a biomarker of tissue damage. CK is composed by two monomers, B and M.\(^2\) Combinations of these monomers generate three isoenzymes CK-BB, CK-MB, and CK-MM, which are found predominantly in brain, heart, and skeletal muscle, respectively.\(^3\) Small amount of CK-BB may be found also in intestine and spleen.\(^5\) In addition to the 3 dimeric isoenzymes, there is a structurally different mitochondrial isoform, CK-MT or CKm, detectable in tissues, and 2 macroenzymes, the macro-CK1 and the macro-CK2, detectable in blood. Macro-CK1 is formed by the dimeric CK-BB bound to the immunoglobulins circulating in the bloodstream, while macro-CK2 consists of oligomers of CK-MT.\(^3\) These two macroenzymes increase in immune-mediated and neoplastic conditions in people,\(^6\) but are present in variable proportion in blood of clinically healthy dogs.\(^4\)

Total CK activity can be measured in plasma or serum using an enzymatic method that utilizes CK-N-acetylcysteine (CK-NAC).\(^7\) Total CK activity mainly reflects CK-MM activity, which accounts for the majority of plasma CK activity in many species.\(^8\) CK-MB, that may be used to diagnose or stage myocardial diseases in people and in some animal species, is usually measured using immunoenzymatic methods.\(^9\) Conversely, most of the methods to determine the activity of CK-BB and macroenzymes are laborious or expensive.\(^3\) Nevertheless, electrophoretic separation has been shown to be a reliable method to identify and quantify CK-BB and macroenzymes in many species.\(^4\,10\,11\)

Due to its prevalent localization within the Central Nervous System (CNS), CK-BB may provide information about CNS lesions. Serum CK-BB activity in dogs with CNS disease was so far investigated in a previous study using a combined immunosubtraction/electrophoretic method.\(^12\)
Recently, a preliminary study based on electrophoretic fractionation demonstrated increases of serum CK-BB activity in 3 dogs with CNS signs. Unfortunately, information about the final diagnosis of CNS diseases in the dogs included in the study cited above was incomplete and it was not possible to establish in which neurological condition CK-BB specifically increases.

The hypothesis of this study is that the type of CNS disorder may influence the activity of CK isoenzymes or macroenzymes in serum, since the degree of neuronal damage is more intense in inflammatory or degenerative lesions than in functional lesions (i.e. idiopathic epilepsy) or when space occupying lesions are present.

Therefore, the aim of this study was to describe the electrophoretic distribution of CK isoenzymes and macroenzymes in serum of dogs with central neurologic disorders, grouped according to disease categories (degenerative, idiopathic, inflammatory, space-occupying lesions) in order to assess whether increases of total CK or of CK iso- and macroenzymes in serum may depend on a specific CNS disease.

Materials and Methods

Medical records of dogs with CNS signs, referred to IPortoni Rossi Veterinary Hospital over a 3-year period, were reviewed periodically to select the samples to be used in this study.

Inclusion in this study required:

- documentation of a complete neurological examination on presentation;
- Magnetic Resonance Imaging (MRI) of either the brain or spinal cord;
- analysis of cerebrospinal fluid (CSF) collected from either cerebellomedullary or lumbar cistern (macroscopic evaluation, total nucleated cell count, total protein concentration, cytological evaluation);
- data regarding complete blood count (CBC), a basic panel of serum biochemical analytes that included urea, creatinine, glucose, total protein, albumin, alkaline phosphatase,
alanine aminotransferase, lactate dehydrogenase, gamma-glutamyl transferase, calcium, phosphorus, serum protein electrophoresis and urinalysis;

- a final diagnosis of idiopathic epilepsy (IE), space occupying lesions (SOL), inflammatory or degenerative disease. The diagnosis of IE was based on unremarkable results of physical and neurological examinations, haematological and serum biochemical analyses, brain MRI, and CSF analysis. The diagnosis of SOL was based on the detection of intra- or extra-axial masses at MRI in dogs without CSF findings consistent with inflammation (see below); we included in this subgroup any lesion that may exert compressive or infiltrative effects on CNS parenchyma, and therefore cases were included in this subgroup even in the absence of a final histological diagnosis. Diagnosis of inflammatory condition was based on course of disease, multifocal CNS signs, inflammatory CSF (protein content > 25 mg/dL, nucleated cell count > 5/µL), and MRI features suggestive of an inflammatory disease: positive tests for infectious diseases further supported the diagnosis of inflammation. Degenerative conditions were diagnosed either presumptively on the basis of history, signalment, results of MRI features and a normal CSF analysis or, in one case, definitively on the basis of histology.

Exclusion criteria were the following:
- grossly hemolyzed or lipemic samples.
- dogs with clinical and/or laboratory changes consistent with metabolic diseases potentially responsible for secondary neurological signs.

Results of test for infectious diseases, necropsy and histopathological examination were recorded, when available.

In addition, ten dogs that were clinically healthy based on a routine physical and neurological examination were selected as control dogs. One additional criterion to include clinically healthy dogs in the control group was the absence of any laboratory abnormality, including a serum activity
of total CK lower than 150 U/L, the reference interval determined in our laboratory on a population of 80 clinically healthy dogs following the ASVCP guidelines for the establishment of reference intervals.\textsuperscript{14} Details of the dogs included in the study are reported in the results section as recommended by the STARD guidelines.\textsuperscript{15}

All dogs were client-owned and sampled for diagnostic purposes or during routine wellness examination. Therefore, according to the regulations of the University of Milan, a formal authorization of the Institutional Animal Care Committee was not necessary.

Venous blood samples were collected into vacutainer plain tubes (Venoject, Terumo Italia Srl, Rome, Italy). Serum was obtained by centrifugation (1100g x 8 min). After collection and separation of serum, total CK activity was measured using the CK-NAC method and an automated spectrophotometer (Cobas Mira, Roche Diagnostic, Basel, Switzerland) with reagents from Real Time Diagnostic System (Viterbo, Italy). The CK-NAC method employs N-acetyl cysteine to attempt to restore the possible loss of CK activity due to reversible oxidation.\textsuperscript{4} Samples were finally stored at -20°C until electrophoretic analyses were performed.

The electrophoretic separation of CK isoenzymes and macroenzymes was performed within one month from samplings on all the samples from healthy dogs and on 40 out of 45 dogs with CNS signs. Five samples were used only to determine total CK activity since the amount of serum available was not sufficient to perform the electrophoretic separation of isoenzymes. A commercially available kit (Hydragel ISO-CK, Sebia Italia) and an automated apparatus (Hydrasys, Sebia Italia) equipped with specific accessories (Standard Mask Accessories for ISO-CK/LD) were used, following manufacturer instructions modified as described in a previous study.\textsuperscript{4} Briefly, 200 \( \mu \)Ls of serum were mixed with 2 \( \mu \)Ls of the activating solution containing \( \beta \)-mercaptoethanol and incubated 10 min at room temperature. Twenty \( \mu \)Ls of this mixture were placed in the applicator.

The gel included in the kit (agarose 8%, pH 8.40 ± 0.05) and the applicator were placed in the
migration chamber and the automatic migration programme was then selected. After migration
(10W to 20W, 27Vh, 20°C) the CK substrate added with the chromogen solution were applied and
the reaction was stopped using the blocking solution. Gels were then washed, dried by heating and
placed on the scanner provided with the instrument. Scanned images were then analyzed using a
specific software (Phoresis, Sebia Italia) and visually inspected in order to correct possible errors of
the automatic separation.

Intra- and inter-assay coefficient of variations (CV), as a measure of the imprecision, as assessed in
a previous study⁴ ranged from 1.4 to 2.9% for the most abundant macro- or isoenzymes (CK-MM,
CK-BB, Macro-CK2), and were slightly higher for CK-MB (intra-assay CV = 3.8%; inter-assay CV
= 9.4%) or for macro-CK1 (7.4% and 12.0% respectively) each of which accounted for less than
2% of total CK activity.

Statistical analyses were done using an Excel spreadsheet (Microsoft Corp, Redmond, WA, USA)
and Analyse-it software (Analyse-it Software Ltd, Leeds, UK). Results from the whole group of
dogs with central neurologic disease were compared with those of the healthy animals using a Mann
Whitney U test. Then, results from healthy dogs and from each subgroup of dogs with CNS disease
were compared to each other using a non parametric ANOVA test (Kruskal Wallis test), followed
by the Bonferroni test as a post-hoc statistical analysis. Both the Mann Whitney U test and the
Kruskal Wallis test use the Tukey’s rule to identify the observations that behave as outliers (near
outliers: values exceeding the III quartile plus 1.5xIQR or the I quartile plus 1.5xIQR; far outliers:
values exceeding the III quartile plus 3.0xIQR or the I quartile plus 1.5xIQR). However, outliers in
single electrophoretic fractions were retained since the comparison of data regarding the other
fractions of the same dogs allowed us to exclude that these aberrant observations were due to
analytical or pre-analytical artifacts). A P value < 0.05 was considered as statistically significant.

Results
Group composition  Forty-five dogs (19 males and 26 females) meeting the inclusion criteria were identified. The median age was 6 years (range, 0.16–16 years). Thirty-five (77.8%) dogs were purebreds, including 6 Boxers, 3 Labrador retrievers, 3 Siberian huskies, 2 Beagles, 2 German shepherd dogs, 2 Jack Russell terriers, and 1 dog of each of the following breeds: Airedale terrier, American cocker spaniel, American Staffordshire terrier, Australian sheepdog, Bernese mountain dog, Cane Corso, Collie, English bulldog, English setter, French bulldog, Greyhound, Kurzhaar, Pinscher, Pointer, Poodle, Samoyed, Weimaraner. The remaining 10 (22.2%) were mixed-breed dogs. Based on the diagnostic work up mentioned above, dogs with CNS disorders were subgrouped as shown in table 1. The ten healthy dogs were 6 males and 4 female, had an age range from 1 to 12 years (median age: 7 years) and included 3 mixed-breed dogs (30%) and 7 (70%) purebreds dogs (2 German shepherd dogs, 2 Labrador retrievers, 1 English setter, 1 Standard schnauzer).

Distribution of CK electrophoretic fractions in serum  Electrophoretic fractionation of CK iso- and macroenzymes using the modified method employed in this study resulted in a clear separation of bands on the gels (figure 1), that in turn led to distinct and narrow peaks on the electrophoretograms (figure 2). Results from healthy dogs and from pathologic dogs are reported in table 2. The activities of total and fractionated CK of healthy dogs were similar to those recorded in a previous study. The predominant electrophoretic fraction was CK-MM, followed by macro-CK2 and CK-BB while CK-MB and macro-CK1 were virtually absent. Compared with results of healthy dogs, the whole group of dogs with CNS disorders had a significantly higher total serum CK activity and CK-BB activity, and a significantly lower Macro-CK2 activity. No significant differences were found for CK-MB, Macro-CK1 or CK-MM activity.
All the parameters of the CNS group, however, were characterized by a high inter-individual variability. Comparison of pathological subgroups and healthy dogs revealed significant differences in dogs with IE and inflammatory disorders for total CK activity, in all the subgroups for CK-BB, and in dogs with IE and SOL for Macro-CK2. The individual variability was high in all the pathological subgroups.

Discussion
The results of this study were consistent with those obtained in a previous study. Specifically, despite the low number of dogs and the relative heterogeneous composition of the pathological subgroups, that are actually limitations of this study, significant increases of some CK isoenzymes, and especially of CK-BB, were detected in dogs with CNS diseases. In almost all cases, the magnitude of these increases was higher than the intrinsic analytical variability of the method reported in the previous study, supporting the hypothesis that increases were dependent on pathological conditions and not on the analytical imprecision of the method. This study demonstrates that the increase of total CK recorded in dogs with CNS diseases does not depend only on the release of CK-MM from muscle cells due to convulsions or to prolonged recumbency, as it has been postulated in the past. Release of CK-MM from skeletal muscle and, to a lesser extent, of CK-MB from myocardium, is probably only one of the events increasing total CK activity. However, the high inter-individual variability, likely depending on the different degree of neurological signs potentially affecting muscle functions, caused these differences to be statistically insignificant. However, the higher median value of CK-MM in dogs with CNS inflammation may in part depend on the muscle activity associated to the seizures (e.g. MUO) or on muscle contracture associated with cervical pain (e.g. steroid responsive meningitis arteritis) that are more frequent and severe in inflammatory conditions than in other CNS diseases. Muscle
contraction may induce lesions to the cell membranes, and subsequent leakage of intracytoplasmic CK. We postulate that the same mechanism may explain, in dogs with inflammation, the increase (also in this case not significant likely due to the high individual variability) of CK-MB (in the case of systolic contraction), or macro-CK2 (oligomers of mitochondrial CK that may be released when cell damage induced by muscle contraction is particularly severe). Conversely, the increase of Macro-CK1, may be a consequence, as in all the other groups, of the increase of CK-BB since Macro-CK1 is a dimer between CK-BB and the antibodies already present in serum. In all other sub-groups apart from the inflammatory group, conversely, macro-CK2 decreases, although this decrease is unexplained and likely not significant on a biological point of view.

The increase of CK-BB was consistently found in all the pathologic subgroups. In human adults, CK-BB is mainly expressed in brain. The increased serum CK-BB activity in dogs with CNS diseases may depend either on the release of this isoenzyme by the cytoplasm of neurons or a damage of the blood-brain barrier. This is not surprising for inflammatory disorders, where cell damage due to inflammatory mediators may induce the leakage of intracytoplasmic enzymes. Theoretically, permeabilization of cell membranes could be responsible for the leakage of CK-BB from neurons also in degenerative conditions and in dogs with SOL, since hypoxic conditions potentially associated with compression exerted by masses may induce permeabilization of cell membranes, similarly to what happens in hepatocytes for transaminases. However, this seems to not occur consistently, since CK-BB was increased only in 2/3 of dogs with SOL (4/6). It would be interesting to verify if the magnitude of CK-BB increases varies in different types of inflammation (e.g. infectious vs. immune-mediated), degenerative diseases or SOL. This was impossible in the current caseload, since the number of dogs per subgroup was too low to perform a reliable statistical comparison. Regarding SOLs, in this study histological diagnosis was available only for 3 out of 4 extra-axial masses and none for intra-axial SOLs. Therefore, statistical comparison was not possible due to the lack of a definite histological diagnosis or low number of cases per subgroup. However,
in future studies it would be interesting to compare the results of dogs with a conclusive histological diagnosis for both intra- and extra-axial SOLs. This comparison may be important since it is likely that the magnitude of changes in total or fractionated CK may be influenced by the type and location of tumors. Similarly, it would be interesting to assess the changes in the activity of CK iso- and macroenzymes in dogs with different degenerative conditions. To this aim, it would be necessary to achieve a final histological diagnosis since in the current study the diagnosis of degenerative conditions has been based on exclusion of other diseases and based on signalment, history, and laboratory investigation. Although, based on these information, it is very unlikely that dogs included in this disease category are not affected by degenerative disorders, a definitive histological diagnosis would improve the possibility to correlate the results with degenerative conditions.

The increase of serum CK-BB in dogs with IE is surprising, since this condition should be characterized by alterations of neuronal functions in the absence of any identified morphological abnormality.\(^\text{17}\) Therefore leakage of CK-BB from neurons would not be expected. It is possible that transient changes of neuronal permeability, or alterations in membrane transporters or receptors not detectable with routine diagnostic imaging or histopathology, may induce the leakage of intracellular enzymes. Finally, it is possible that some of these patients suffered from status epilepticus (SE), that by definition is characterized by seizures lasting more than five minutes or by a series of at least two discrete seizures without full recovery of consciousness between the seizures.\(^\text{18}\) Continuous seizure activity of 30 minutes or longer may cause systemic dysfunction, including hypoxia, altered blood pressure, and hyperthermia and can lead to temporary or permanent brain lesions.\(^\text{17}\) Unfortunately, it was not possible to standardize the time between the first appearance of clinical signs and the time of sampling, since samples were collected during the routine diagnostic activity of a referral center for neurological diseases. It would be interesting, in
the future, to investigate whether the increase of CK-BB activity in serum of dogs with IE depends on the presence and severity of SE.

Independently of the mechanisms responsible for the increase of CK-BB, from a diagnostic or prognostic standpoint, the increase of CK-BB may be useful to confirm a diagnosis of CNS disease. However, CK-BB increases in all the pathological sub-groups studied and it does not differentiate among different CNS diseases. Theoretically, the inflammatory diseases might be differentiated from others CNS diseases, since they had multiple abnormalities in CK isoenzymes and macroenzymes (e.g. a simultaneous increase of CK-BB, CK-MM, Macro-CK1). Although these changes are not statistically significant in terms of group comparison, the magnitude of these increases was often very high. Therefore, an increase of one or more of these parameters above a given threshold may support a clinical diagnosis of inflammatory CNS disorder. Definition of this threshold, however, should be based on an appropriate statistical approach. This approach cannot be used in the present study, since our caseload included only healthy dogs and dogs with CNS disorders and not all the non-neurological conditions characterized by increases of one or more isoenzymes (e.g. myopathy, cardiomyopathy, muscular or neurological damage secondary to a series of primary pathological conditions, etc). Therefore, the specificity of any change would result artifactually inflated since the number of false positive result detectable in routine practice (i.e. results above a given threshold in dogs not affected by primary CNS disease) is not available. However, this aspect merits further investigation in the future. Additionally, it would important also to quantify, on a larger cohort of dogs, the sensitivity of changes in total and fractioned CK since the data reported in table 2 evidence an overlapping between the results of sick dogs and results of healthy dogs, which in turn were within the reference interval of the laboratory. The quantification of these “false negative” results may allow us to better understand the diagnostic accuracy of increased total and fractioned CK. Similarly, further studies are needed to clarify whether the quantification of CK-BB in cerebrospinal fluid (CSF) may have a clinical utility. In this study, no
CK activity was recorded in four frozen and thawed CSF samples (data not shown) and also the
electrophoretic separation of iso- or macroenzymes did not reveal visible bands on these samples.
This is likely a storage artifact since it is known that many enzyme activities decrease rapidly in
frozen CSF\textsuperscript{20} while the negative effect of freezing-thawing on serum samples, although present,\textsuperscript{21} is
minimal. A decrease of total CK accounting for approximately 10\% has been reported in sera stored
for up to 6 months\textsuperscript{21} while the visualization of bands is not affected by storage at -20°C.\textsuperscript{4} Therefore,
storage would have minimally affected the possibility to quantify total CK or electrophoretic bands
in serum.\textsuperscript{4} Conversely, measurement of total CK activity and electrophoretic analysis of CK iso-
and macro-enzymes should be performed on fresh CSF samples or after a preliminary concentration
step to assess its actual practical utility.

In conclusion, despite some limitations such as the low number of animals per group and the huge
individual variability, this study demonstrated that many CK isoenzymes, including CK-BB,
increase in serum of dogs with CNS diseases. This increase seems not to depend on the type of
disease, and it is likely due to the leakage of CK-BB from neurons affected by inflammatory or
degenerative conditions or to the hypoxia associated with space occupying lesions. The mechanism
responsible of increases of CK-BB in dogs with idiopathic epilepsy, remains to be elucidated. From
a diagnostic standpoint, the measurement in serum of total CK and of CK iso- or macroenzymes
(with special emphasis on CK-BB) may be sufficient to confirm suspected CNS disease when,
based on the clinical presentation, the pre-test probability of CNS disorders is high. However, this
increase by itself cannot identify the specific neurologic disorder, so further diagnostic testing such
as diagnostic imaging or CSF analysis is still necessary to help classify specific disease.

Additionally, future studies should include also a group of dogs with non neurological diseases and
with increased total CK to assess whether CK-BB is truly specific for CNS diseases. The design of
this study did not allow us to define whether the increase of one or more iso- or macroenzyme may
have a prognostic utility in predicting the outcome of the CNS diseases. This aspect, as well as the
possible mechanism responsible for the leakage of CK-BB merits to be further investigated in future studies.

Acknowledgments

This study was partially funded by a 2008 F.I.R.S.T. grant from the University of Milan. The authors thank Dr. Johnatan Ongaro for his support

Conflict of interest

The Authors do not have conflicts of interest regarding this study

References


Table 1: Type of diseases recorded in dogs with neurological signs included in this study.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>DIAGNOSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerative disease (n=7)</td>
<td>senile atrophy (n=2)</td>
</tr>
<tr>
<td></td>
<td>degenerative myelopathy (n=2)</td>
</tr>
<tr>
<td></td>
<td>storage disease / hereditary degeneration (n=2)</td>
</tr>
<tr>
<td></td>
<td>ceroid lipofuscinosis (n=1, histologically confirmed)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic epilepsy (n=14)</td>
<td></td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Inflammatory disorders (n=16)</td>
<td>meningoencephalitis of unknown origin (MUO - n=9)</td>
</tr>
<tr>
<td></td>
<td>steroid responsive meningitis-arteritis (SRMA - n=4)</td>
</tr>
<tr>
<td></td>
<td>idiopathic bilateral trigeminal neuritis (n=1)</td>
</tr>
<tr>
<td></td>
<td>cerebral abscess (n=1)</td>
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<tr>
<td></td>
<td>neosporosis (n=1)</td>
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<td></td>
<td></td>
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<tr>
<td>Space occupying lesions (SOL)</td>
<td>intraaxial masses (n=4)</td>
</tr>
<tr>
<td>(n=8)</td>
<td>extraaxial masses (n=4, 3 of which histologically confirmed as tumors)</td>
</tr>
</tbody>
</table>
Table 2. Mean ± S.D., median (between brackets) and minimum-maximum activities of total and fractionated CK (U/L) in serum from healthy dogs and from subgroups of dogs with central neurologic diseases. Total CK activity was determined by the CK-NAC method.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group/subgroup</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Healthy dogs (n=10)</td>
</tr>
<tr>
<td></td>
<td>Whole group (n=40)</td>
</tr>
<tr>
<td>CK tot‡‡</td>
<td>56 ± 23</td>
</tr>
<tr>
<td></td>
<td>(58) 17-96</td>
</tr>
<tr>
<td>CK-BB‡‡</td>
<td>12 ± 9</td>
</tr>
<tr>
<td></td>
<td>(9) 2-29</td>
</tr>
<tr>
<td>CK-MB</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(0.1) 0.0-0.7</td>
</tr>
<tr>
<td>Macro-CK1</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>(0.2) 0.0-1.7</td>
</tr>
<tr>
<td>----------------</td>
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<tr>
<td><strong>CK-MM</strong></td>
<td>34 ± 17</td>
</tr>
<tr>
<td></td>
<td>(34) 11-69</td>
</tr>
<tr>
<td><strong>Macro-CK2†‡</strong></td>
<td>9 ± 6</td>
</tr>
<tr>
<td></td>
<td>(6) 3-19</td>
</tr>
</tbody>
</table>

* n=45 for total CK activity; †† n=16 for total CK activity; † † † n=8 for total CK activity; † † † † ANOVA (healthy vs. pathological subgroups) = P<0.01; * P < 0.05 compared with healthy dogs; ** P < 0.01 compared with healthy dogs; *** P < 0.001 compared with healthy dogs.
Figure captions:

Figure 1: example of electrophoretic gel that includes a control material (C) composed by a mixture of homogenized brain, cardiac and skeletal muscle of dogs and cats as specified in Paltrinieri et al.\textsuperscript{4} and sera from a clinically healthy dog (H), and from dogs with neurological diseases classified as degenerative (D), idiopathic epilepsy (IE), inflammation (I) and space occupying lesions (SOL).

Figure 2: example of electrophoretograms obtained in a clinically healthy dog (H) and in dogs with neurological diseases classified as degenerative (D), idiopathic epilepsy (IE), inflammation (I), and space occupying lesions (SOL).

Figure 3: Box and whiskers histograms showing the distribution of total CK and CK fraction activities (U/L) for healthy dogs (H) and from subgroups of dogs with central neurologic disease (Deg = degenerative; IE = idiopathic epilepsy; I = inflammation; SOL = space occupying lesions). The boxes indicates the I–III interquartile range (IQR), the horizontal line indicates the median values, whiskers extend from the I quartile minus 1.5xIQR to the III quartile plus 1.5xIQR. The open circles indicates near outliers (values exceeding the III quartile plus 1.5xIQR); the black dots indicates far outliers (values exceeding the III quartile plus 3.0xIQR). Black bolded symbols within the boxes indicate significant differences versus healthy dogs (**P < 0.001).