Spurious capillary zone electrophoresis pattern in hypercholesterolemic dogs

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Abstract. Capillary zone electrophoresis (CZE) is a relatively new serum protein electrophoresis (SPE) method with higher resolution than other electrophoretic techniques. Hypercholesterolemic dogs exhibit a peculiar CZE pattern. Specifically, they have a shoulder or peak immediately next to the albumin peak. We investigated the prevalence of this spurious peak in hypercholesterolemic dogs and its correlation with the serum cholesterol concentration. Moreover, possible discrepancies between the CZE and spectrophotometric (bromocresol green method; BCG) albumin concentrations in those animals were evaluated, as well as the accuracy in measuring albumin by a different CZE fractionation system. We retrospectively enrolled 500 hypercholesterolemic and normotriglyceridemic dogs. Each electrophoretic curve was inspected visually to identify a spurious peak (prevalence of 68.8%). We chose 120 dogs to further investigate the albumin concentration; CZE albumin was significantly higher than measured using the BCG method. A weak but significant correlation ($r = 0.412$; $p < 0.0001$) was observed between the magnitude of the spurious peak and the serum cholesterol concentration. Finally, the significant difference between CZE and BCG albumin measurement disappeared ($p = 0.92$) when the spurious peak was considered as α1 globulins instead of albumin.

Keywords: albumin; canine; capillary zone electrophoresis; cholesterol; lipoproteins.

Serum protein electrophoresis (SPE) is considered a reference method for the evaluation of serum protein classes (namely albumin, and α1, α2, β, and γ globulins).[26] SPE is used widely in veterinary medicine for research and diagnostic purposes, as well as to monitor treatment. Indeed, variations in protein fraction concentrations, together with an abnormal electrophoretic pattern, can help to identify various pathologic conditions, such as acute and chronic inflammation, infectious diseases,[19,43] and neoplasia.[17,30] Moreover, it can help to identify immunodeficiencies that are characterized by low levels of immunoglobulins, which usually migrate in β- and γ-globulin regions.[8] Furthermore, in canine species, electrophoretic flattening is considered a positive prognostic factor in evaluation of the treatment response in chronic infectious diseases, such as leishmaniasis and ehrlichiosis.[8]

Three electrophoretic methods can be used to evaluate serum proteins: cellulose acetate, agarose gel (AGE), and capillary zone (CZE) electrophoresis. The CZE system is a relatively new technique that has been validated for dogs and cats.[19] Even though CZE is more expensive than the other methods, it offers some advantages, such as higher resolution,[17] high degree of automation, and similar sensitivity to AGE in detecting monoclonal peaks.[21]

In SPE, albumin is the most prominent peak on the anodal side of the electrophoretogram,[40] and its concentration can be quantified by converting its electrophoretic percentage from the total protein concentration measured by the biuret method.[32] However, albumin concentration can also be measured using spectrophotometric methods. Indeed, the bromocresol green (BCG) method is a dye-binding method that is used routinely to measure the albumin concentration in both human[20] and veterinary medicine.[16] Nevertheless, the BCG dye is not an albumin-specific reagent; thus, it can also bind other serum proteins, such as the acute-phase proteins,[20] leading to false overestimation of the albumin concentration, which seems more severe in hypoalbuminemic dogs and horses.[35] Overestimation of the albumin

58 concentration was reported as well using the BCG method in heparinized canine plasma
59 samples because of fibrinogen binding.[44] In human medicine, overestimation has been
60 reported for the CZE albumin concentration compared to bromocresol purple and
61 immunoturbidimetric methods.[14,32] However, there are no similar reports in veterinary
62 medicine, probably because of the difficulties to run nephelometry and immunoturbidimetry,
63 which are considered the gold standard methods for serum albumin measurement.[14,32]
64 Hypoalbuminemia is a common type of dysproteinemia,[26] and the accuracy of serum albumin
65 concentration measurement is pivotal, especially to allow recognition and severity assessment
66 of hypoalbuminemia, as well as for monitoring purposes.
67 Cholesterol, the main sterol in animals, can be obtained from a diet based on animal
68 products or it can be synthesized, mostly by the liver, endocrine glands, and other tissues.
69 Pure cholesterol and cholesterol esters are hydrophobic and insoluble in plasma, and they
70 must be transported as lipoproteins. Plasma lipoproteins are very large molecules composed
71 of lipids, phospholipids, and proteins,[6] and they can be classified according to their
72 electrophoretic mobility (α–β lipoprotein regions) or, more frequently, according to their
73 chemical and physical aspects (density, size, composition) determined by ultracentrifugation,
74 in which case, they are classified as chylomicrons, very low-density lipoproteins (VLDL),
75 low-density lipoproteins (LDL), and high-density lipoproteins (HDL).[26] Chylomicrons are the
76 main transporters of dietary lipids and serum triglycerides; VLDL, LDL, and HDL mainly
77 contain cholesterol and are involved in the metabolism of endogenously produced lipids.[4,45,47]
78 Some diseases (e.g., nephrotic syndrome) or comorbidities (e.g., cholestasis associated
79 with inflammation) can lead to concurrent hypoalbuminemia and hypercholesterolemia.
80 Indeed, it is pivotal to recognize any possible interference of hypercholesterolemia with the
81 serum albumin concentration measurement to avoid clinical misclassification.[11]
82 Hypercholesterolemia, as well as the presence of different lipoprotein profiles, have been

investigated in dogs.[45,47,49] Primary hyperlipidemia is reported in various breeds, such as Briards,[46] Rough Collies,[22] Shetland Sheepdogs,[39] and Miniature Schnauzers.[38,48] Endocrine disorders, such as hypothyroidism,[37] diabetes mellitus, and hyperadrenocorticism,[18] as well as other diseases, such as protein-losing nephropathy,[24] cholestasis,[12] and obesity,[10,31] are among the most frequent conditions associated with hypercholesterolemia.

During routine analyses in our diagnostic laboratory (BiEsseA Laboratorio Analisi Veterinarie, Milano, Italy), we observed a shoulder on the right side of the albumin peak (cathodal side) of serum CZE in dogs with hypercholesterolemia. To our knowledge, this electrophoretic pattern has not been reported previously in veterinary medicine. In human medicine, a cathodic shoulder on the albumin peak using CZE is reported with severe hyperlipidemia, especially with hypertriglyceridemia.[7,36] In veterinary medicine, interference as a result of hypertriglyceridemia has been reported as a cathodic peak in the α2-globulin region.[28] The inclusion of this spurious peak within the albumin fraction could cause albumin overestimation and consequent underestimation of the globulin fractions, with increased risk of clinical misclassification.

Our aims were 1) to assess the prevalence of this spurious electrophoretic pattern in hypercholesterolemic dogs, 2) to compare CZE and BCG albumin measurement in hypercholesterolemic dogs, 3) to investigate correlation between the observed spurious CZE peak and serum cholesterol concentration, and 4) to propose an electrophoretic fractionation system to improve the accuracy of CZE albumin measurement in hypercholesterolemic dogs.

Materials and methods

Sample selection

We retrospectively searched the database of the commercial veterinary laboratory BiEsseA Laboratorio Analisi Veterinarie, from November 2019 to March 2021, for 500 canine serum samples with the following inclusion criteria:

- Availability of serum biochemistry, performed using an automated spectrophotometer (AU 480; Beckman Coulter) and including the following parameters: urea, creatinine, calcium, potassium, sodium, chloride, glucose, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, creatine kinase, lactate dehydrogenase, gamma-glutamyl transferase, amylase, lipase, total bilirubin, total protein (TP), albumin, globulin, albumin:globulin ratio, cholesterol, triglycerides, C-reactive protein.
- Serum triglyceride concentration (measured using the glycerol phosphate oxidase method) within-the-laboratory RIs (WRIs; 0.34–1.24 mmol/L).
- Serum cholesterol concentration (measured using the cholesterol esterase method) higher than the laboratory upper RI limit (URL; >7.51 mmol/L).
- SPE evaluated on fresh serum samples and performed by a CZE automated analyzer (Minicap; Sebia) using reagents provided by the manufacturer (Protein(E) 6 kit) with standard setting.

As part of our QA procedure, we monitored the reproducibility of results from 2 reference canine serum samples obtained from routine submissions and stored properly (with normal and pathologic electrophoretic patterns, respectively) run together with routine samples.[1] Specifically, instrument analytical performance was judged as acceptable if CVs were <10% for each electrophoretic fraction. The CZE curves were inspected visually by 2 qualified operators (S Rossi, ECVCP Diplomate; G Mangiagalli, ECVCP resident) for a spurious peak on the cathodic side of the albumin peak. Both operators were blinded to the sample cholesterol concentration. The spurious peak might appear as an inflection point of variable amplitude and shape (Fig. 1).

From the 500 samples selected, we selected 120 more-recent cases with the spurious peak (group A) and used them to assess the correlation between the magnitude of the spurious albumin peak and the concentration of cholesterol, as well as for comparison with BCG-

measured albumin. As well, we selected 50 serum samples from a control population (group B), which included normocholesterolemic and normotryglyceridemic dogs selected in the same retrospective period as group A, according to the following inclusion criteria:

- Availability of the same serum biochemical parameters reported for group A.
- Serum triglycerides concentration WRI 0.34–1.24 mmol/L.
- Serum cholesterol concentration WRI 3.62-7.51 mmol/L.
- SPE performed using the same CZE automated analyzer as for group A. CZE curves were visually evaluated for the absence of the spurious peak.

For each serum sample, TP values (measured using the biuret method) and BCG albumin, globulins (calculated by subtraction of albumin from TP), cholesterol, and triglycerides were retrieved from the biochemistry panel. The concentrations (g/L) of the spurious electrophoretic peak (when present) and of the other protein fractions were calculated using the electrophoresis software (Phoresis; Sebia) based on the TP concentration and the percentage of each fraction, calculated as the area under the curve (AUC) of each electrophoretic peak. Then, each electrophoretic curve from group A was modified manually to separate the spurious peak from the major albumin peak at the inflection point. The spurious peak was then included in the α1-globulin fraction, and the AUC of the modified albumin and the combined spurious peak plus α1-globulin fractions were automatically recalculated by the CZE instrument software.

Statistical analysis

Statistical analyses were performed (Analyse-it v.4.97 software) in Excel (v.2209, Microsoft). The distribution of data (cholesterol and albumin measured with both BCG and CZE) was assessed through the Shapiro-Wilk test. Because the distribution of data was not normal for group A, subsequent analyses were performed using non-parametric statistical tests. The correlation between BCG and CZE albumin in both groups, and the correlation between

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158 serum cholesterol and the spurious peak AUC in group A, was performed using the Spearman
159 test. The comparison between the Δalbumin (i.e., the difference between albumin measured
160 using CZE and BCG) in the 2 groups was performed using the Wilcoxon-Mann-Whitney *U*
161 test. The same test was used to compare the results of the α1-globulin concentration before
162 and after modification of the electrophoretogram in group A. The agreement between
163 methods (BCG vs CZE) for the evaluation of albumin concentration was performed using
164 Passing-Bablok regression analysis and Bland-Altman difference plot testing in both groups.
165 Statistical significance was set at $p \leq 0.05$.

166 **Results**

167 Among the canine samples with hypercholesterolemia enrolled retrospectively, 344 of 500
168 (68.8%) had a spurious CZE peak; the remaining 156 dogs had hypercholesterolemia without
169 a spurious electrophoretic peak. Moreover, dogs with a spurious peak had a higher median
170 cholesterol concentration (median: 9.32 mmol/L; min–max: 7.50–17.92 mmol/L) compared to
171 those without a spurious CZE peak (median: 8.39 mmol/L; min–max: 7.50–11.74 mmol/L; p
172 <0.0001).

173 Based on the inclusion criteria, we further evaluated the most recent 120 of the 344
174 hypercholesterolemic dogs with the spurious peak (group A). Purebred dogs (n = 85; 70.8%)
175 were mainly Golden Retrievers (n = 16; 13.3%) and German Shepherds (n = 6; 5%); the
176 remaining dogs were crossbred dogs (n = 35, 29.2%). Of these 120 dogs, 52 (43.3%) were
177 females and 65 (54%) were males (sex was not reported for 3 dogs); neutered or intact status
178 was not reported. The median age was 8.8 y (range 0.4–18 y). The control group (group B, n
179 = 50), had a breed composition similar to that of group A (34 purebred, 68%; 16 crossbred,
180 32%). Females were 30 (60%); males were 20 (40%) (sex was not reported for 4 dogs);
181 neutered or intact status was not reported. The median age was 6.8 y (range 0.5-14 y). None
182 of the dogs belonging to group B had a spurious electrophoretic peak.

There was no statistical difference in albumin concentration between groups A and B measured with either BCG ($p = 0.68$) or CZE ($p = 0.052$; Table 1). Both groups had a strong significant positive correlation between CZE albumin and BCG albumin concentrations, which was higher in group B ($r = 0.933$; $p < 0.0001$) than in group A ($r = 0.881$; $p < 0.0001$).

Passing-Bablok regression analysis of the albumin methods calculated for group A revealed a proportional bias when including the spurious peak within the albumin fraction (intercept: -0.12; 95% CI: -3.58–2.87; slope: 1.23; 95% CI: 1.12–1.34; Fig. 2). The Bland–Altman test highlighted significantly higher values of CZE albumin when including the spurious peak within the albumin fraction (bias 6.5; 95% CI: 6.08–6.91; $p < 0.0001$; Fig. 2). For group B, Passing-Bablok regression analysis of the albumin methods revealed proportional bias (intercept: -0.47; 95% CI: -4.49–3.98; slope: 1.17; 95% CI: 1.04–1.29; Suppl. Fig. 1). The Bland–Altman test highlighted significantly higher values of CZE albumin compared to the BCG method (bias 4.65; 95% CI: 4.22–5.07; $p < 0.0001$; Suppl. Fig. 1). However, the Δalbumin was significantly higher in group A than in group B (group A median: 6.4 g/L; min–max: -1.6–15.4 g/L; group B median: 4.9 g/L; min–max: 1–7.2 g/L; $p < 0.0001$).

A weak but significant correlation was observed between the spurious peak (expressed in g/L) and the cholesterol concentration in group A ($r = 0.412$; $p < 0.0001$). On the contrary, no significant correlation was observed between the spurious peak and BCG albumin ($r = 0.094$; $p = 0.30$) or TP ($r = 0.108$; $p = 0.24$) concentrations.

Removing the spurious peak from the CZE albumin fraction resulted in a slightly increased correlation between BCG albumin and corrected CZE albumin concentration in group A ($r = 0.894$; $p < 0.0001$). Passing-Bablok regression analysis of the albumin methods revealed both a constant and a proportional bias after the removal of the spurious peak from the CZE albumin fraction in group A (intercept: -6.31; 95% CI: -10.24–-3.66; slope: 1.21;

95% CI: 1.12–1.34; Fig. 2). Conversely, the Bland–Altman test highlighted the absence of significant differences between CZE and BCG albumin measurements when removing the spurious peak from the albumin fraction (bias -0.02, 95% CI: -0.4–0.36; $p = 0.92$; Fig. 2).

The spurious peak was then included in the α1-globulin fraction, resulting in a significant increase of the latter fraction (original α1 median: 3.3 g/L; min–max: 1.7–6.8 g/L; α1 + spurious peak median: 9.7 g/L; min–max: 7.1–14.5 g/L; $p < 0.0001$). Specifically, observing the original data, only 6 of 120 dogs (5%) had an α1-globulin concentration that exceeded the URL for this specific fraction, whereas the inclusion of the spurious peak into the α1-globulin peak resulted in a concentration of this fraction above the URL in all dogs enrolled in group A. In group A, 35 of 120 dogs (42%) were hypoalbuminemic according to BCG results. However, considering the original data using CZE, only 9 of 120 dogs (10.8%) were classified as hypoalbuminemic. After removal of the spurious peak, 31 of 120 (37.2%) dogs had an albumin value that fell below the lower reference limit (LRL), meaning that inclusion of the spurious peak in the albumin fraction would result in misclassification of 22 dogs (26.4%). Some discordant results were observed between CZE and BCG. Specifically, 4 dogs were classified as hypoalbuminemic with CZE but normoalbuminemic with BCG (however, all of the BCG results were close to the LRL). Eight dogs were classified as hypoalbuminemic with BCG but as normoalbuminemic using CZE even after removal of the spurious peak.

Discussion

We observed a peculiar electrophoretic pattern with a spurious peak located in the cathodic side of the albumin fraction in 344 of 500 (68.8%) hypercholesterolemic dogs, but never in normocholesterolemic dogs. The observed correlation between the spurious peak concentration and serum cholesterol concentration may suggest that cholesterol (most likely HDL cholesterol) is the main component of this peak, even though other possible components

of the spurious peak could not be excluded completely. It is worth noting that, in our prevalence study, some hypercholesterolemic dogs did not have the spurious peak, thus suggesting that a different lipoprotein-bound cholesterol (namely HDL and LDL) could possibly affect the presence and the shape of the peak. Indeed, the spurious peak could appear in different shapes, even in samples with similar serum cholesterol concentrations. In some cases, it appeared as a cathodic shoulder on the albumin peak, whereas in others it was a more defined and isolated peak. Further studies, investigating the lipoprotein composition in hypercholesterolemic dogs with and without the spurious CZE albumin peak, may help in elucidating these aspects. These differences may rely on different lipoprotein migration properties based on their classes (e.g., VLDL, LDL, HDL), or on the classes involved in a specific pathologic process. Indeed, it is well known that lipoprotein classes and their concentrations can vary depending on the underlying disease.[49]

In our caseload, CZE albumin of dogs with the spurious peak was significantly higher than BCG albumin. Given that BCG may have low specificity in measuring serum albumin in dogs,[44] the observation of higher CZE albumin values compared to BCG increased the suspicion of a possible overestimation of CZE albumin, most likely the result of increased cholesterol concentrations, leading to an inaccurate CZE albumin measurement. Thus, a correction of this electrophoretic alteration might be needed to obtain a more reliable albumin concentration in these patients. To reduce the interference of cholesterol with CZE albumin measurement, we considered different fractionation systems. Complete removal of the spurious peak was considered inappropriate given that it would lead to rearrangement of the electrophoretic fraction concentrations with subsequent overestimation of the globulin fractions (Suppl. Table 1). For this reason, we preserved the spurious peak and included it in the α1-globulin fraction; regardless of the electrophoretic method used, this region is not considered important diagnostically.[23] In healthy dogs, α1 globulins are represented by a

258 weak band or a flat region between the albumin and the α2-globulin fraction, and it is
259 assumed that α1 lipoprotein (also called HDL), α1 antitrypsin, and α1 antichymotrypsin
260 migrate in this region.[42]

261 Note that most of the information about the contribution of specific proteins to the
262 different electrophoretic regions is based on human medicine. Indeed, studies about CZE α1
263 fraction protein migration are lacking in veterinary medicine, and it has been simply
264 presumed that the protein migration may be similar to that reported in people. In human
265 medicine, α1 lipoprotein may overlap the albumin fraction (Sebia Capillarys) or may appear
266 as a diffuse increase between albumin and α1 acid glycoprotein (orosomucoid) band (Paragon
267 CZE 2000; Beckman Coulter), depending on the analyzer.[23,25] We used a Minicap analyzer
268 (Sebia) in our study, and, according to the manufacturer, α1 lipoprotein, which is the most
269 prominent lipoprotein fraction reported in dogs,[9,27] could migrate as part of the albumin band
270 as it does in human samples. The use of specific methods, such as immunoelectrophoresis[15,41]
271 or lipoprotein electrophoresis,[5,29] would allow identification of the proteins that migrate in the
272 spurious peak. However, given the retrospective nature of our study, it was not possible to
273 perform specific additional evaluations. Nevertheless, based on reports in people, a major
274 component of this peak could likely be represented by α1 lipoproteins in
275 hypercholesterolemic dogs. Another hypothesis that would explain the presence of the
276 spurious peak in hypercholesterolemic dogs could be that the binding between serum
277 cholesterol and albumin may give a different electrophoretic mobility to the albumin itself. In
278 human medicine[34] and in rats,[13] it has been hypothesized that serum cholesterol (especially
279 the non-esterified form) could be a serum albumin ligand, acting as a transport protein. Thus,
280 the spurious peak could be considered a portion of the albumin fraction with different
281 migration properties; similarly, it has been reported that drugs and hormones bound to
282 albumin can lead to a different migration pattern of the albumin itself in SPE.[2,3] However, if

283 the spurious peak was composed of molecule-bound albumin, the removal of this peak from
284 the albumin fraction would possibly have resulted in a negative bias in CZE albumin
285 compared to BGE albumin. On the contrary, the removal of the spurious peak from the
286 albumin fraction resulted in better agreement, supported by the Bland-Altman test, with no
287 significant differences between CZE and BCG albumin measurement, making the hypothesis
288 of albumin-bound cholesterol less likely.

289 A positive bias has been reported in the measurement of human serum albumin
290 concentration with CZE compared to bromocresol purple, turbidimetry, and nephelometry
291 methods.[32,33] In the control group of our study (group B), the comparison of the 2 methods
292 revealed a significant difference between CZE and BCG albumin concentrations, with higher
293 CZE albumin concentrations. In dogs, it was suggested that the serum albumin concentration
294 measured with the BCG method tended to be higher compared to SPE.[35] However, in our
295 study, the serum albumin concentration was lower when measured with the BCG method
296 compared to CZE in control dogs. Further studies should be performed to better investigate
297 this result. However, given our results, it is advisable to use the same method when
298 comparing serial albumin measurements (e.g., for treatment monitoring).

299 One of our aims was to evaluate the agreement between CZE and BCG methods for
300 the measurement of the albumin concentration. The inclusion of the spurious peak in the α1
301 fraction instead of the albumin fraction increased the correlation between CZE and BCG
302 albumin concentration, and the Bland–Altman test revealed the absence of significant
303 differences between these methods. Thus, the proposed electrophoretogram modifications
304 would decrease the risk of clinical misclassification of the albumin concentration using CZE
305 in hypercholesterolemic dogs, improving the agreement and correlation between CZE and
306 BCG albumin concentrations. The risk of misclassification was further supported observing
307 the noteworthy number of group A dogs (15 of 120) that would have been erroneously

considered as normoalbuminemic rather than hypoalbuminemic if the spurious peak had been included in the albumin fraction. Moreover, the inclusion of the spurious peak among α1 globulins avoids the rearrangement of the globulin fractions, given that the α1 region was the only fraction affected. Nevertheless, the clinical relevance of an increase of the α1 fraction has not been reported in veterinary medicine.

Our study has some limitations. Lipoprotein electrophoresis and immunoelectrophoresis would be suggested to confirm our hypothesis about the presence of lipoproteins and to evaluate which classes of lipoproteins migrate in the albumin region in CZE in dogs. Moreover, we performed only the CZE method in our study. The use of different electrophoretic methods or instruments could increase the robustness of the results obtained. Even though no reports are present in the literature about spurious peaks in hypercholesterolemic dogs using AGE, investigations are warranted to verify if the higher albumin concentrations in hypercholesterolemic dogs found in our study can be detected also using other SPE techniques. Another limitation is that a standardized cutoff allowing isolation of the spurious peak was not feasible, and removal of the spurious peak was performed only on visual inspection of the curves. This is because of both the different shapes of the CZE albumin peak in normal samples[19] and also the different appearances of the spurious peak in hypercholesterolemic dogs. Our approach, especially in samples with only an albumin right-shoulder spurious peak, may lead to false, slightly lower, CZE albumin concentrations, given that the manual cutoff could include a very small portion of the albumin peak. Nevertheless, our results highlighted better correlation between the 2 albumin measurements after removal of the spurious peak, thus supporting the methodology employed. Discordant results should be interpreted cautiously, especially in those cases in which only CZE albumin was lower than the RIs (considering that, in healthy animals, CZE albumin concentrations were higher than BCG albumin). Laboratory results should always be interpreted considering the history

and other clinical findings. Finally, although we investigated the agreement between 2 methods that are used routinely to measure the albumin concentration, neither of these is the gold standard for albumin measurement. Nevertheless, the use of immunoturbidimetry or nephelometry is not feasible in routine testing, given the need for species-specific antibodies.

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References

1. Arnold JE, et al. ASVCP Guidelines: Principles of Quality Assurance and Standards for Veterinary Clinical Pathology (version 3.0): Developed by the American Society for Veterinary Clinical Pathology's (ASVCP) Quality Assurance and Laboratory Standards (QALS) Committee. Vet Clin Pathol 2019;48:542-618.
2. Arvan DA, et al. Transient “bisalbuminemia” induced by drugs. Clin Chim Acta 1968;22:211- 218.
3. AvRuskin TW, et al. Familial dysalbuminemic hyperthyroxinemia: a rare example of albumin polymorphism and its rapid molecular diagnosis. J Pediatr Endocrinol Metab 2002;15:801-807.
4. Bauer JE. Comparative lipid and lipoprotein metabolism. Vet Clin Pathol 1996;25:49–56.
5. Behling-Kelly E. Comparison of 2 electrophoretic methods and a wet-chemistry method in the analysis of canine lipoproteins. Vet Clin Pathol 2016;45:124–134.
6. Bruss ML. Lipids and ketones. In: Kaneko JJ, et al., eds. Clinical Biochemistry of Domestic Animals. 6th ed. Elsevier, 2008: 81–116.

7. Bossuyt X, et al. Automated serum electrophoresis by Capillarys. Clin Chem Lab Med 2003;41:704–710
8. Ceron JJ, et al. Answers to some common questions on serum protein electrophoresis. Vet Rec. 2011;168:453-454.
9. Chapman MJ. Comparative analysis of mammalian plasma lipoproteins. In: Segrest JP, Albers JJ, eds. Plasma Lipoproteins Part A: Preparation, Structure, and Molecular Biology. Methods in Enzymology. Elsevier, 1986;128:70-143.
10. Chikamune T, et al., Serum lipid and lipoprotein concentrations in obese dogs. J Vet Med Sci 1995;57:595–598.
11. Conner BJ. Treating hypoalbuminemia. Vet Clin North Am Small Anim Pract 2017;47(2):451-459.
12. Danielsson B, et al. Plasma lipoprotein changes experimental cholestasis in the dog. Clin Chim Acta 1977;80:157-170.
13. Deliconstantinos G, et al. Evidence for the existence of non-esterified cholesterol carried by albumin in rat serum. Atherosclerosis 1986;61:67-75.
14. Duly EB, et al. Measurement of serum albumin by capillary zone electrophoresis, bromocresol green, bromocresol purple, and immunoassay methods. J Clin Pathol 2003;56:780-781.
15. Dumon MF, et al. Discontinuous polyacrylamide gel - agarose gel immunoelectrophoresis for analysis of plasma lipoproteins. Electrophoresis 1990;11:846-885.
16. Evans GO, Parsons CE. A comparison of two dye-binding methods for the determination of dog, rat and human plasma albumins. J Comp Pathol 1988;98:453-460.
17. Facchini R, et al. Detection of biclonal gammopathy by capillary zone electrophoresis in a cat and a dog with plasma cell neoplasia. Vet Clin Pathol 2010;39:440–446.

18. Feldman EC, Nelson RW. Canine and Feline Endocrinology. 4th ed. Saunders, 2015:77–554.
19. Giordano A, Paltrinieri S. Interpretation of capillary zone electrophoresis compared with cellulose acetate and agarose gel electrophoresis: reference intervals and diagnostic efficiency in dogs and cats. Vet Clin Pathol 2010;39:464–473.
20. Hill PG. The measurement of albumin in serum and plasma. Ann Clin Biochem 1985;22:565-578.
21. Jeffries CM, Harris RA, Ashton L, Moore, AR. Method comparison for serum protein electrophoretic M-protein quantification: agarose gel electrophoresis and capillary zone electrophoresis in canine and feline sera. Vet Clin Pathol 2021;50:543–550
22. Jeusette I, et al. Hypercholesterolaemia in a family of rough collie dogs. J Small Anim Pract 2004;45:319–324.
23. Karen DF. Proteins identified by serum protein electrophoresis. In: Keren DF, ed. Protein Electrophoresis in Clinical Diagnosis. 2nd ed. Edward Arnold, 2003:69-71.
24. Klosterman ES, Pressler BM. Nephrotic syndrome in dogs: clinical features and evidence-based treatment considerations. Top Companion Anim Med 2011;26:135–142.
25. Luraschi P, et al. High-density lipoproteins contribute to the alpha-1-globulin zone in capillary electrophoresis of human serum proteins. Electrophoresis 2004;25:1551–1554.
26. Eckersall PD. Serum protein, proteomics, and dysproteinemias. In: Kaneko JJ, et al., eds. Clinical Biochemistry of Domestic Animals. 6th ed. Elsevier, 2008:117–155.
27. Mahley RW, et al. Canine lipoproteins and atherosclerosis. II. Characterization of the plasma lipoproteins associated with atherogenic and nonatherogenic hyperlipidemia. Circ Res 1974;35:722-733.

406 28. Martínez-Subiela S, et al. Effects of haemolysis, lipaemia, bilirubinaemia and
407 fibrinogen on protein electropherogram of canine samples analysed by capillary zone
408 electrophoresis. Vet J 2002;164:261-268.
409 29. Milanovic Z, et al. Association of acute *Babesia canis* infection and serum lipid,
410 lipoprotein, and apoprotein concentrations in dogs. J Vet Intern Med 2019;33:1686–1694.
411 30. Moore AR, Avery PR. Protein characterization using electrophoresis and
412 immunofixation; a case-based review of dogs and cats. Vet Clin Pathol 2019;48:29–44.
413 31. Mori N, et al. Potential use of cholesterol lipoprotein profile to confirm obesity status
414 in dogs. Vet Res Commun 2011;35:223–235.
415 32. Padelli M, et al. Is capillary zone electrophoresis a suitable method for estimating
416 serum albumin: a comparison of four methods. Clin Chim Acta 2018;487:250–255.
417 33. Padelli M, et al. Systematic overestimation of human serum albumin by capillary zone
418 electrophoresis method due to monoclonal immunoglobulin interferences. Clin Chim Acta
419 2019;491:74–80.
420 34. Peng L, et al. The interaction between cholesterol and human serum albumin. Protein
421 Pept Lett 2008;15:360-364.
422 35. Ramery E, Bureau F. Measurement of serum albumin by protein electrophoresis and
423 bromocresol green methods in canine and equine patients. Vet Clin Pathol 2012;41:5.
424 36. Regeniter A, Siede WH. Peaks and tails: evaluation of irregularities in capillary serum
425 protein electrophoresis. Clin Biochem 2018;51:48–55.
426 37. Rogers WA, et al. Lipids and lipoproteins in normal dogs and in dogs with secondary
427 hyperlipoproteinemia. J Am Vet Med Assoc 1975;166:1092–1100.
428 38. Rogers WA, et al. Idiopathic hyperlipoproteinemia in dogs. J Am Vet Med Assoc
429 1975;166:1087–1091.

430 39. Sato K, et al. Hypercholesterolemia in Shetland Sheepdogs. J Vet Med Sci
431 2000;62:1297–1301.
432 40. Snozek CL,et al. Comparison of bromcresol green and agarose protein electrophoresis
433 for quantitation of serum albumin in multiple myeloma. Clin Chem. 2007;53:1099-1103.
434 41. Solyom A, et al. Apolipoprotein and lipid distribution in canine serum lipoproteins.
435 Biochim Biophys Acta 1971;229:471-483.
436 42. Stockham SL, Scott MA. Proteins. In: Stockham SL, Scott MA, eds. Fundamentals of
437 Veterinary Clinical Pathology. 2nd ed. Blackwell, 2008:378.
438 43. Stranieri A, et al. Frequency of electrophoretic changes consistent with feline
439 infectious peritonitis in two different time periods (2004-2009 vs 2013-2014). J Feline
440 Med Surg. 2017;19:880-887.
441 44. Stokol T, et al. Overestimation of canine albumin concentration with the bromcresol
442 green method in heparinized plasma samples. Vet Clin Pathol 2001;30:170- 176.
443 45. Watson TDG, Barrie J. Lipoprotein metabolism and hyperlipidaemia in the dog and
444 cat: a review. J Small Anim Pract 1993;34:479-487.
445 46. Watson P, et al. Hypercholesterolaemia in Briards in the United Kingdom. Res Vet Sci
446 1993;54:80–85.
447 47. Xenoulis PG, Steiner JM. Lipid metabolism and hyperlipidemia in dogs. Vet J
448 2010;183:12-21.
449 48. Xenoulis PG, et al. Investigation of hypertriglyceridemia in healthy Miniature
450 Schnauzers. J Vet Intern Med 2007;21:1224–1230.
451 49. Xenoulis PG, Steiner JM. Canine hyperlipidaemia. J Small Anim Pract 2015;56:595–
452 605.

453

454 **Table 1.** Median and minimum–maximum values for albumin (g/L) measured both with
455 bromocresol green (BCG) and capillary electrophoresis (CZE) in hypercholesterolemic dogs
456 with the spurious electrophoretic peak (group A) and in normocholesterolemic dogs without
457 the spurious electrophoretic peak (group B). No significant differences were observed in
458 albumin concentration between groups A and B measured with either BCG or CZE.

Method	Group	Minimum	Median	Maximum
BCG	A	16.0	29.8	36.7
	B	18.0	29.6	41.3
CZE	A	18.2	36.6	48.2
	B	21.3	34.8	45.0

459

Figure 1. Examples of capillary zone electrophoresis (CZE) patterns in normocholesterolemic and hypercholesterolemic dogs, with different spurious peak shapes. **A.** CZE of a normal canine serum sample without a spurious peak (cholesterol concentration: 4.39 mmol/L). **B.** Right shoulder within the albumin peak (cholesterol concentration: 9.82 mmol/L). **C.** Isolated peak on the right side of the albumin peak, (cholesterol concentration: 9.26 mmol/L). **D.** Distinct isolated peak on the right side of the albumin peak (cholesterol concentration: 18.67 mmol/L).

Figure 2. Passing-Bablok regression analyses (left) and Bland-Altman difference plots (right) of albumin measurement with capillary zone electrophoresis (CZE) compared to the bromocresol green (BCG) method in hypercholesterolemic dogs with a spurious CZE peak **A)** before and **B)** after the removal of the spurious albumin peak.

Supplemental Figure 1. Passing-Bablok regression analysis (left) and Bland-Altman plot (right) of albumin measurement with capillary electrophoresis (CZE) compared to the bromocresol green (BCG) method in normocholesterolemic dogs without the spurious electrophoretic peak (group B).

Commentato [mgm3]: Looks fine now. I uploaded.

Commentato [mgm4]: The plots are OK, but please move A and B to the lower left corner of the images, and reset ALBUMIN as albumin.
Thanks.

Commentato [SM5R4]: Both Figure 2 and S1 have been modifies as suggested (there is a slight modification in height of Figure 2 to have enough space for A and B in the lower left corner, I hope this may be fine).

Commentato [mgm6]: Same comment for albumin

Commentato [SM7R6]: Done (see above)