Effect of Sample Time in Clot Tube, Temperature, and Hemolysis Status on Canine Serum Brain-Derived Neurotrophic Factor (BDNF) Concentrations: Preliminary study

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a neurotrophin which promotes a variety of neural processes ranging from neuronal health to the survival in the peripheral and central nervous systems. There is interest in serum BDNF as a potential biomarker of anxiety disorders in pet dogs for veterinary and comparative behavior research. As an initial step in applying BDNF in canine research, work was performed to determine appropriate sample conditions for BDNF analyses. Objectives of this study was to determine the effects of blood storage time in clot tube, temperature, and hemolysis on measured BDNF concentrations in dog serum. Blood was collected from dogs (n=11) with no evidence of behavioral or neurological conditions, and BDNF serum concentrations measured by ELISA. The following conditions were evaluated for effects on BDNF serum concentrations: time of blood in clot tube (time from placing blood in clot tube until centrifugation/serum collection, 30 minutes, 2 hours, 24 hours), temperature (4°C, 25 °C) and extent of hemolysis (non, moderate, marked). BDNF concentrations were not affected by time in clot tube, temperature, or sample hemolysis. In conclusions, this study demonstrates the sampling conditions that can be used when measuring BDNF concentrations in dogs, and expands the clinical settings for use of this test.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a neurotrophin which promotes a variety of neural processes ranging from neuronal health to the survival in the peripheral and central nervous systems.[1] BDNF passes the blood-brain barrier, thus blood concentrations of BDNF are expected to reflect brain concentrations of BDNF.[2] Indeed, positive correlations between BDNF in brain tissue and peripheral blood concentrations have been shown in humans, rodents and pigs.[2-4] Based on the role and function BDNF plays in the central nervous system, there is considerable interest in using BDNF as a biomarker in anxiety disorders, and there is evidence for this in rodents.[5] In humans, peripheral blood BDNF concentrations were lower in patients with obsessive-compulsive disorder and depression compare to non-affected subjects.[6, 7] In humans with depression, BDNF concentrations have increased after patients were treated with antidepressants such as selective serotonin reuptake inhibitors, tricyclic antidepressants, serotonin norepinephrine reuptake inhibitors, and tetracyclic antidepressants.[8-10] In a meta-analysis,[11] it was concluded that BDNF could potentially serve as a peripheral blood biomarker to aid in the diagnosis of psychiatric disorders as well as to monitor the efficacy of treatment for mood disorders. Our group is especially interested in BDNF with the goal to identify biomarkers to use in diagnosing and monitoring canine behavior disorders, such as separation anxiety. It is essential to development better strategies to detect and manage separation anxiety in dogs as it is the most common anxiety disorder in dogs, and it often leads to the dog being relinquished or euathanized.[12] Another reason to study anxiety disorders in dogs is to define mechanisms and better strategies for interventions that could possibly apply to humans, as well as dogs. [13] To date, limited studies have published regarding canine BDNF concentrations. Reports have included studies of BDNF in old dogs such as a correlation between canine brain BDNF concentrations and cognitive performance,[14] and change of serum BDNF concentrations before and after antioxidant dietary supplement in aged dogs.[15]

The most common method of measuring BDNF concentrations in serum, plasma, and platelets from rats and humans is Enzyme-Linked-Immunosorbent-Assay (ELISA).[16, 17] Blood platelets are the main reservoirs of blood BDNF, from which it can be released during platelet activation or coagulation processes, and therefore serum BDNF concentration are 10 times higher than plasma concentrations.[16] Serum BDNF concentrations are considered more relevant in anxiety studies. There are various factors in humans that can affect peripheral blood BDNF concentrations including age, weight, fasting state, drinking, smoking, exercise level, and living environment.[18-20] Additionally, it has been suggested that pre-analytical factors, such as sample handling and storage conditions, can affect peripheral blood BDNF concentrations. However, it is unclear how these pre-analytical factors affect BDNF, and the results from studies and among species have been inconsistent.[20-25] For example, Maffioletti et al. (2014) reported that in human samples at constant temperature, serum BDNF concentrations continued to increase at 10 minutes, and 30 minutes of clotting time till up to 1 hour of clotting time.[23] Another study reported that BDNF was affected by the clotting temperature, and degraded unless the sera were kept at 4^{o}C.[20]

Another factor that could potentially affect peripheral blood BDNF concentrations is the degree to which a serum sample is hemolyzed. Hemolysis can occur due to mishandling the collection of blood in a clinical setting. Hemolysis can prevent analyzers from measuring the absorbance of light as a result of a color reaction, which is used to accurately quantify BDNF.[26] To date, published studies of the effects of hemolysis on serum BDNF concentrations have been lacking.

With the goal to apply BDNF analysis in research and subsequent clinical application

in dogs, it is critical to validate the sampling conditions that will allow accurate test results. The aim of this study was to compare various pre-analytical factors including blood storage time in clot tube, temperature, and extent of sample hemolysis when using a commercially available Canine BDNF ELISA kit.

MATERIALS AND METHODS

All work was approved by the Purdue Animal Care and Use Committee (protocol no. 1501001179). Blood was collected from healthy dogs with no evidence of behavioral or neurologic conditions following consent from the dog owner.

Effects of Sample Time in Clot Tube and Temperature on BDNF Concentrations:

Blood samples were collected and aliquoted into 6 clot tubes. Three tubes were kept at room temperature (25°C), and three tubes at 4◦C. Samples kept at 25°C were allowed to sit in the clot tubes for 30 minutes, 1 hour, and 2 hours (one tube/condition). The samples kept at 4°C were allowed to sit in the clot tubes for 30 minutes, 1 hour, and 24 hours (one tube/condition). After meeting the specific time point, each tube was centrifuged at approximately 15,000 g for 15 minutes at 4°C. All serum samples were stored at -80°C until the BDNF measurement took place.

The BDNF assay was performed within two months of sample collection.

The BDNF concentrations were measured by a commercially-available Canine BDNF ELISA (Cloud-Clone Corp., Houston, TX, USA) following manufacturers protocol. The absorbance was measured using a microtiter plate reader at 450 nm (Molecular Devices, CA, Sunnyvale, CA, USA). The concentration of BDNF in the samples was expressed as nanograms of BDNF per milliliter (ng/mL) of serum using an external BDNF standard curve provided with the ELISA. According to the manufacturer, the detection range of the BDNF ELISA kit is 0.156 -10 ng/mL, and the sensitivity is 0.061 ng/mL.

Effect of Hemolysis on BDNF Concentrations:

Non-hemolyzed blood samples were obtained and placed in clot tubes. Following clotting for 1hour at room temperature, the tubes were centrifuged as above, and serum collected and stored at -80°C. Hemolysis conditions were experimentally produced as previously described.[27] Briefly, specific hemoglobin concentrations were achieved by adding washed canine red blood cells (Innovative Research, Inc., Novi, MI, USA) in a serum pool.[27] Three hemoglobin concentrations were prepared, that is, non-hemolysis (0 g/L), moderate hemolysis (2.5 g/L), and marked hemolysis (10 g/L), by adding the washed canine red blood cells to the 10 non-hemolyzed sera. BDNF concentrations were measured as described above.

Statistical Analyses

Blood samples from a minimum of 10 dogs were included to evaluate clot tube time, temperature, and hemolysis conditions. The effects of sample time in the clot tube and temperature were evaluated using Friedman's test and Wilcoxon signed ranks test.

To evaluate the effect of hemolysis on canine serum BDNF concentrations, BDNF concentrations for the three hemolysis concentrations were compared using a linear mixed model. The statistical significance for all analyses was set at $P < 0.05$, and the analyses were performed using IBM SPSS Statistics for Windows (Version 22.0, Armonk, NY: IBM Corp).

RESULTS

To evaluate the effect of time in clot tube and temperature on canine serum BDNF concentrations, serum samples from 11 clinically healthy dogs were used. Among these dogs were 5 purebred and 6 mixed breed dogs. The median age was 5.7 years old (range: 1-11 years). All of the dogs (4 females and 7 males) had been neutered or spayed. To evaluate the effect of hemolysis on canine serum BDNF concentrations, 10 serum samples were used including samples from 8 purebred and 2 mixed breed dogs.

Table 1. *Median (range) serum BDNF concentrations (ng/mL) at different clot tube storage conditions*

	Sample time in clot tube			
Temperature	30 minutes	1 hour	2 hours	24 hours
4°C	0.232 (0.042-3.193)	0.141 (0.048-3.267)		0.543 (0.050-2.783)
Room Temperature (25°C)	0.141 (0.055-2.068)	0.110 (0.061-2.168)	0.128 (0.055-4.043)	

Their median age was 5.9 years old (range: 1-10 years). Nine of the dogs had been neutered or spayed (4 females and 5 males) and 1 dog was an intact male.

The serum BDNF concentrations at different clot tube times and temperatures are summarized in Table 1. The change in serum BDNF concentrations over clot tube times in each temperature in each dog were shown at Figure 1 and Figure 2. There were no significant differences in BDNF concentrations among the three groups (30 minutes, 1 hour and 2 hours) at room temperature ($p = 0.913$) or at 4°C ($p = 0.148$). No significant differences in BDNF concentrations were observed between the two temperatures at 30 minutes (room temperature, 4°C) ($p = 0.091$) or at 1 hour ($p = 0.722$). Additionally, there were no significant differences observed in BDNF concentrations between the 2-hour clot tube time at room temperature and the clot tube time at 24 hours at 4°C ($p = 0.374$).

The mean serum BDNF concentrations for each hemolysis level are shown in Figure 3. For non-hemolyzed and marked hemolyzed samples, samples from 10 dogs were analyzed. For moderate hemolyzed samples, serum from 6 dogs were analyzed; insufficient sample volume precluded moderate hemolysis samples being generated in the other 4 dogs. There were no significant differences in BDNF concentrations among the three hemolysis concentrations ($p = 0.273$)

DISCUSSION

In order to support behavioral medicine's exploration of serum BDNF as a potential biomarker for canine behavioral disorders such as separation anxiety, this study investigated effects of the pre-analytical

Figure 1. *Change in BDNF concentration (ng/mL) in different clot tube times at room temperature*

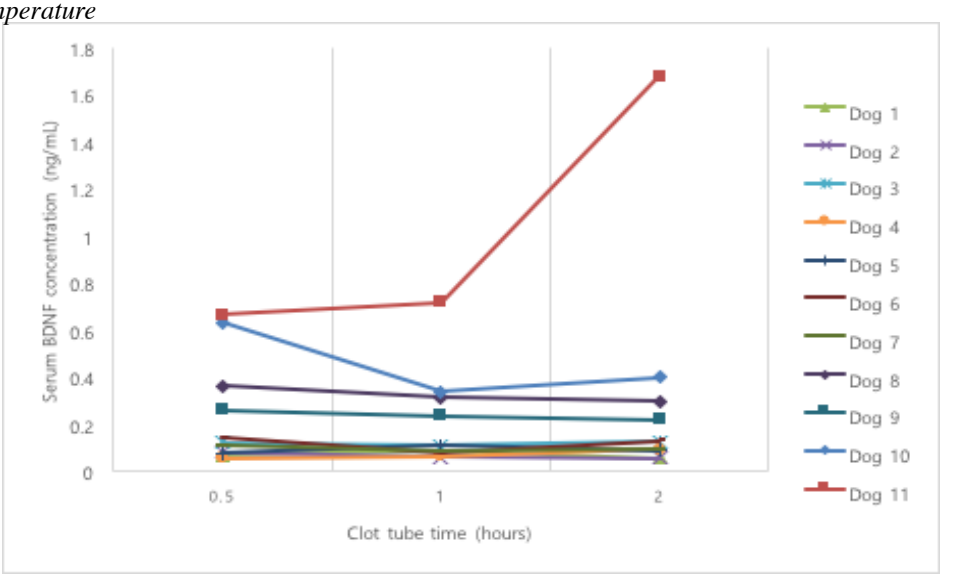
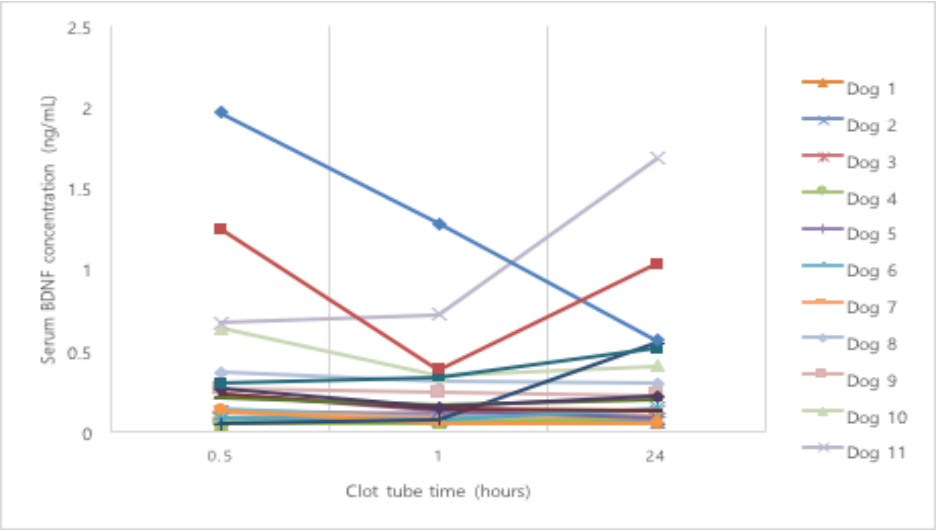
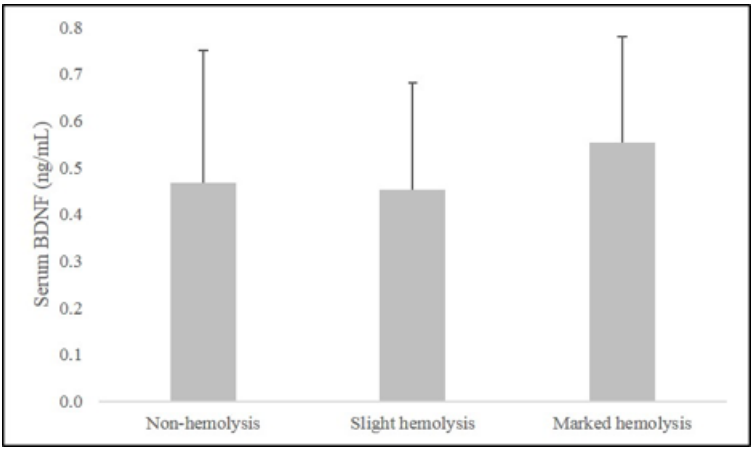


Figure 2. *Change in BDNF concentration (ng/mL) in different clot tube times at 4°C*



conditions such as sample time in clot tube, temperature, and sample hemolysis on canine serum BDNF concentrations. The results showed that there was no statistical difference between the clot tube time and the temperature in canine serum BDNF concentrations at the times and temperatures used. Measured concentrations were very consistent in 9 of the 11 dogs, although variation in measured BDNF concentrations were noted in dogs #10 and 11. While these dogs were thought to be healthy, it is not known if they could have an occult underlying condition that could affect platelet coagulation and potentially BDNF concentrations. Our overall results were slightly different from those of a previous study in humans which reported that a significant difference was observed between 30 min and 1 hour of clot tube time at room temperature, although the same study reported that the serum concentration of BDNF at 30 minutes clot tube time at room temperature reached 91.8% of the BDNF concentrations at 1 hour or longer clot tube time at room temperature[23]. No statistical differences were observed in canine serum BDNF concentrations between clot tube time at room temperature and at 4°C for a duration of 30 minutes, or for 1 hour, which does not agree with the conclusion of a previous study in human samples[20]. Although it was small sample size, the subjects in the current study were compared to themselves therefore; sex, age and individual signalment were all controlled in our study. Figure 1 and Figure 2 showed BDNF concentrations in the majority individuals were

Figure 3. *Mean canine serum BDNF concentration (ng/mL) for different hemolysis conditions. Error bars are standard deviation*



stable, and the range of the concentrations were similar over time except for dog 10 and dog 11. Dogs 4, 10 and 11 were the only dogs that were older than 8 years old in this samples, and it is unknown if their age affected their BDNF concentrations. Further study with larger sample size would be needed to confirm this.

Even though the results from our study showed that the clot tube time and temperature did not significantly affect the BDNF concentrations in dogs, it would still be advisable to apply a consistent protocol of clot tube time and temperature that is manageable in the clinical setting for further study in dogs.

In the second part of the work, canine serum BDNF concentrations among three different hemolysis levels were compared. A previous study[28] emphasized the importance of pre-analytical conditions in clinical chemistry analyses. Specifically, hemolysis could decrease the quality of the laboratory test result if the assay measures the amount of absorbance of light, such as is the case with ELISA analyses.[26] The results of our study, however, showed no statistical differences among the three different hemolysis concentrations. Findings from this study provide essential information needed in the application of BDNF analyses in behavioral studies in dogs. In conclusion, this study showed that clot tube storage time, temperature and sample hemolysis that are of concern in a clinical setting did not usually affect canine serum BDNF concentrations. The current study contributes the essential information that will lead to further investigation of serum BDNF as a potential biomarker in dogs.

DECLARATION OF CONFLICTING INTERESTS

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

REFERENCES

1. Aydemir C, Yalcin ES, Aksaray S, Kisa C, Yildirim SG, Uzbay T, Goka E. Brain-derived neurotrophic factor (BDNF) changes in the serum of depressed women. *Prog Neuropsychopharmacol Biol Psychiatry.* 2006; 30:1256–1260.
2. Karege F, Schwald M, Cisse M. Postnatal developmental profile of brain-derived neurotrophic factor in rat brain and platelets. *Neurosci Lett.* 2002; 328:261–264.
3. Karege F, Perret G, Bondolfi G, Schwald M, Bertschy G, Aubry J-M. Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry Res.* 2002; 109:143–148.
4. Klein AB, Williamson R, Santini MA, Clemmensen C, Ettrup A, Rios M, Knudsen GM, Aznar S. Blood BDNF concentrations reflect brain-tissue BDNF levels across species. *Int J Neuropsychopharmacol Off Sci J Coll Int Neuropsychopharmacol* CINP. 2011; 14:347–353.
5. Rasmusson AM, Shi L, Duman R. Downregulation of BDNF mRNA in the hippocampal dentate gyrus after re-exposure to cues previously associated with footshock. *Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol.* 2002; 27:133–142.
6. Suliman S, Hemmings SMJ, Seedat S. Brain-Derived Neurotrophic Factor (BDNF) protein levels in anxiety disorders: systematic review and meta-regression analysis. Front Integr Neurosci. 2013; 7:55. Available at: http://dx.doi.org/10.3389/fnint.2013.00055. Accessed July 08, 2013.
7. Molendijk ML, Spinhoven P, Polak M, Bus B a. A, Penninx BWJH, Elzinga BM. Serum BDNF concentrations as peripheral manifestations of depression: evidence from a systematic review and meta-analyses on 179 associations (N=9484). *Mol Psychiatry*. 2014; 19:791–800.
8. Huang T-L, Lee C-T, Liu Y-L. Serum brain-derived neurotrophic factor levels in patients with major depression: effects of antidepressants. *J Psychiatr Res*. 2008; 42:521–525.
9. Piccinni A, Marazziti D, Catena M, Domenici L, Del Debbio A, Bianchi C, Mannari C, Martini C, Da Pozzo E, Schaiavi E, Mariotti A, Roncaglia I, Palla A, Consoli G, Giovannini L, Massimetti G, Dell'Osso L. Plasma and serum brain-derived neurotrophic factor (BDNF) in depressed patients during 1 year of antidepressant treatments. *J Affect Disord.* 2008; 105:279–283.
10. Yoshimura R, Kishi T, Hori H, Katsuki A, Sugita-Ikenouchi A, Umene-Nakano W, Atake K, Iwata N, Nakamura J. Serum Levels of Brain-Derived Neurotrophic Factor at 4 Weeks and Response to Treatment with SSRIs. *Psychiatry Investig*. 2014; 11:84–88.
11. Polyakova M, Stuke K, Schuemberg K, Mueller K, Schoenknecht P, Schroeter ML. BDNF as a biomarker for successful treatment of mood disorders: a systematic & quantitative meta-analysis. *J Affect Disord*. 2015; 174:432–440.
12. Overall KL, Dunham AE, Frank D. Frequency of nonspecific clinical signs in dogs with separation anxiety, thunderstorm phobia, and noise phobia, alone or in combination. *J Am Vet Med Assoc*. 2001; 219:467–473.
13. Ogata N, Gillis TN, Liu X, Cunningham SM, Lowen SB, Adams BL, Sutherland-Smith J, Mintzopoulos D, Janes AC, Dodman NH, Kaufman MJ. Brain structural abnormalities in Doberman

pinschers with canine compulsive disorder. *Prog. Neuropsychopharmacol Biol Psychiatry*. 2013; 45:1-6.

14. Fahnestock M, Marchese M, Head E, Pop V, Michalski B, Milgram WN, Cotman CW. BDNF increases with behavioral enrichment and an antioxidant diet in the aged dog. *Neurobiol Aging*. 2012; 33:546–554.
15. Sechi S, Chiavolelli F, Spissu N, Di Cerbo A, Canello S, Guideti G, Fiore F, Cocco R. An antioxidant dietary supplement improves brain-derived neurotrophic factor levels in serum of aged dogs: preliminary results. *J Vet Med.* 2015; 412501:1-9.
16. Fujimura H, Altar CA, Chen R, Nakamura T, Nakahashi T, Kambayashi J, Sun B, Tandon N. Brain-derived neurotrophic factor is stored in human platelets and released by agonist stimulation. *Thromb Haemost*. 2002; 87:728–734.
17. Watanabe K, Hashimoto E, Ukai W, Ishii T, Yoshinaga T, Ono T, Tateno M, Watanabe I, Shirasaka T, Saito S, Saito T. Effect of antidepressants on brain-derived neurotrophic factor (BDNF) release from platelets in the rats. *Prog Neuropsychopharmacol Biol Psychiatry.* 2010; 34:1450–1454.
18. Bus BAA, Molendijk ML, Penninx BJWH, Buitelaar JK, Kenis G, Prickaerts J, Elzinga BM, Oude Voshaar RC. Determinants of serum brain-derived neurotrophic factor. *Psychoneuroendocrinology.* 2011; 36:228–239.
19. Lommatzsch M, Zingler D, Schuhbaeck K, Schloetcke K, Zingler C, Schuff-Werner P, Virchow JC. The impact of age, weight and gender on BDNF levels in human platelets and plasma. *Neurobiol Aging*. 2005; 26:115–123.
20. Pareja-Galeano H, Alis R, Sanchis-Gomar F, Cabo H, Cortell-Ballester J, Gomez-Cabrera MC Lucia A, Vina J. Methodological considerations to determine the effect of exercise on brain-derived neurotrophic factor levels. *Clin Biochem*. 2015; 48:162–166.
21. Elfving B, Plougmann PH, Wegener G. Detection of brain-derived neurotrophic factor (BDNF) in rat blood and brain preparations using ELISA: pitfalls and solutions. *J Neurosci Methods*. 2010; 187:73–77.
22. Katoh-Semba R, Wakako R, Komori T, Shigemi H, Miyazaki N, Ito H, Kumagai T, Tsuzuki M, Shigemi K, Yoshida F, Nakayama A. Age-related changes in BDNF protein levels in human serum: differences between autism cases and normal controls. *Int J Dev Neurosci Off J Int Soc Dev Neurosci*. 2007; 25:367–372.
23. Maffioletti E, Zanardini R, Gennarelli M, Bocchio-Chiavetto L. Influence of clotting duration on brain-derived neurotrophic factor (BDNF) dosage in serum. *BioTechniques*. 2014; 57:111–114.
24. Trajkovska V, Marcussen AB, Vinberg M, Hartvig P, Aznar S, Knudsen GM. Measurements of brain-derived neurotrophic factor: methodological aspects and demographical data. *Brain Res Bull.* 2007; 73:143–149.
25. Tsuchimine S, Sugawara N, Ishioka M, Yasui-Furukori N. Preanalysis storage conditions influence the measurement of brain-derived neurotrophic factor levels in peripheral blood. *Neuropsychobiology.* 2014; 69:83–88.
26. Meinkoth JH, Allison RW. Sample collection and handling: getting accurate results. *Vet Clin North Am Small Anim Pract*. 2007; 37:203–219, v.
27. Martínez-Subiela S, Cerón JJ. Effects of hemolysis, lipemia, hyperbilirrubinemia, and anticoagulants in canine C-reactive protein, serum amyloid A, and ceruloplasmin assays. *Can Vet J Rev Vét Can*. 2005; 46:625–629.
28. Lippi G, Guidi GC, Mattiuzzi C, Plebani M. Preanalytical variability: the dark side of the moon in laboratory testing. *Clin Chem Lab Med* CCLM FESCC. 2006; 44:358–365.