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Amyloid-beta plasma and cerebrospinal fluid biomarkers in aged dogs with cognitive dysfunction syndrome

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Abstract

Background: Cognitive dysfunction syndrome (CDS) is a common progressive neuro-degenerative disease that is poorly defined. Specific multitargeted protocols do not exist for setting the diagnosis and the prognosis of the syndrome.

Hypothesis/Objectives: To quantify $A\beta42$ and $A\beta40$ peptides in blood and cerebro-spinal fluid (CSF) and to investigate their contribution to CCDS.

Animals: A total of 61 dogs from a hospital population.

Methods: Case-control study. Six young (YG: 0-4 years old), 8 middle-aged (4-8 years old), 17 cognitively unimpaired and aged (CU: 8-20 years old), and 30 cognitively impaired and aged (CI: 8-17 years). From the CI group, 10 dogs exhibited mild impair-ment (CI-MCI) and 20 exhibited severe impairment (CI-SCI). Cognitive status was assessed using a validated owner-based questionnaire. Direct and indirect $A\beta$ markers were determined in plasma fractions (total-TP, free-FP, bound to plasma components-CP) and CSF using commercial ELISA assays (ABtest, Araclon Biotech).

Results: $TPA\beta42/40$ facilitated discrimination between CI-MCI and CU aged dogs with area under curve ≥ 0.79. $CSFA\beta42$ levels were higher ($P = .09$) in CU (1.25 ± 0.28 ng/mL) than in MCI (1.04 ± 0.32 ng/mL) dogs. CSF $A\beta42$ levels were correlated with the CP fragment ($CPA\beta40$: $P = .02$, $CPA\beta42$: $P = .02$). $CPA\beta42$ was higher in the CI-MCI (23.03 ± 11.79 pg/μL) group compared to the other aged dogs (CU: 10.42 ± 7.18 pg/μL, $P = .02$, SCI: 11.40 ± 12.98 pg/μL, $P = .26$).

Conclusion and Clinical Importance: The $A\beta$ should be determined in all of the 3 plasma fractions (TP, FP, CP). In the clinical approach, $TPA\beta42/40$ could be used as an efficient preselection tool for the aged canine population targeting dogs with mild cognitive impairment.

Abbreviations: $A\beta$, amyloid beta; AD, Alzheimer's disease; CAWEC, Companion Animal Welfare Education Center; CDS, cognitive dysfunction syndrome; CI, cognitively impaired; CP, components of plasma; CSF, cerebrospinal fluid; CU, cognitively unimpaired; FP, free plasma; MA, middle aged; MCI, mild cognitive impairment; SCI, severe cognitive impairment; TP, total plasma; YG, young dogs.

1 | INTRODUCTION

Geriatric veterinary medicine is becoming increasingly important as a form of medical care for companion animals, which has led to the prolongation of their life expectancy.[1] Canine cognitive dysfunction syndrome (CDS) is a highly prevalent condition that affects 14% to 60% of aged dogs; however, less than 2% are actually diagnosed.[1-6]

Canine cognitive dysfunction syndrome is a progressive neurodegenerative syndrome that affects dogs older than 8 years with representing signs associated with the gradual and progressive loss of cognitive functions. Disorientation, changes in sleep-wake cycles, social interaction disturbances, loss of house training and other learned behaviors, activity variations, and increased anxiety are the main indicators of the disease in dogs.[7-9]

Currently, no specific protocol exists for the diagnosis of CDS in clinical practice. Moreover, most veterinarians use only owner-based questionnaires, which could lead to misreporting, misinterpretation, and an inability to accurately stage the disease. In contrast, in human medicine biomarker development has improved phenotyping of senile dementia. Senile dementia encompasses different distinct neuropathological conditions, with Alzheimer's disease (AD) being the most common.[10,11] Cerebrospinal fluid (CSF) biomarkers are among the most accurate assessment methods.[12] Blood-based biomarkers are also attractive because they are easily accessible, cost-effective, and scalable.[13] Regarding the similarity of early AD and -CDS, it is understood that the inclusion of this field of biomarkers in veterinary medicine would allow advances to be made in the management of CDS.[14]

The incorporation of a multitargeted approach wherein cognitive tasks assessment is accompanied by the monitoring of related measurements in blood, CSF, or both will improve knowledge of CDS.

Amyloid β protein (Aβ) depositions detected as senile plaques (SP) and cerebral amyloid angiopathy are associated with aging in both dogs and humans and are considered one of the main pathological factors for both CDS and AD.[15,16] Aβ peptides are 38 to 43 amino acids in length that emerge from the enzymatic cleavage of amyloid precursor protein (APP).[17] Aβ40 and Aβ42 are major components of the accumulated Aβ and the most common species found in AD and CDS.[15,17] Aβ-related biomarkers may be optimal for the early staging of AD.[18,19] Considering the 100% homology of the canine Aβ molecule with the human counterpart, the quantification of Aβ fragments in canine blood and CSF can be performed; but, they have not been measured in combination.[20-24] Regarding plasma, Aβ40 and Aβ42 are already measured by using 2 different dilution methods.[14,24]

The aim of the present study was to measure all direct and indirect fractions of Aβ40 and Aβ42 in blood and CSF, in an attempt to improve current knowledge regarding the biochemical changes occurring in different clinical stages of this syndrome. Another goal of the study was the assessment of these biomarkers as potential diagnostic and prognostic tools in the prevention and early management of CDS.

2 | MATERIALS AND METHODS

2.1 | Study population

A total of 61 dogs were selected for the study. These were admitted to the Clinic of Companion Animals, either referred for behavioral consultation or other medical problems. The dogs belonged to various breeds and were treated according to European legislation on animal handling and experiments (86/609/EU). The study was approved by the Ethical Committee of the Veterinary School, Aristotle University of Thessaloniki, Greece (72/25-10-16). The owners of the dogs signed a statement of informed consent for participation in the study.

The animals were allocated into 4 groups: (1) young (YG: 0-4 years old, n = 6); (2) middle-aged (MA: 4-8 years old, n = 8); (3) cognitively unimpaired and aged (CU: 8-20 years old, n = 17); (4) Cognitively impaired and aged (CI: 8-17 years old, n = 30). A subdivision of the CI group was further made to ensure a more comprehensive and in-depth evaluation of the issue. Specifically, this group was separated into 2 groups on the basis of the behavioral test score of animals (Table 1); the first group included dogs with mild cognitive impairment (CI-MCI), while the second group included dogs with severe cognitive impairment (CI-SCI).

The Canine Cognitive Assessment Scale (Table 1) from Landsberg et al,[8] modified by the Companion Animal Welfare Education Center (CAWEC), was used for the evaluation of mental activity in aged dogs and their subsequent allocation in the aforementioned groups.[7,8] As proposed by the CAWEC group, the questionnaire was completed by the dog owners with assistance from the medical staff to help explain the questions. Before inclusion in the study, all dogs were screened extensively with clinical, neurological, and clinicopathological examinations (complete blood counts, serum biochemistry, urinalysis) in order to exclude other medical or behavioral problems that could mimic CDS signs.

2.2 | Blood and CSF sampling and Aβ peptide analysis

Blood samples were collected in K-EDTA polypropylene vials from the jugular vein and were processed as previously described.[24] Briefly, they were centrifuged (2.500g, 4°C, 15 minutes) within 2 hours of collection to harvest the plasma, which was then aliquoted (3 aliquots per patient) and immediately frozen at −80°C. CSF was collected, centrifuged, (2.000g, 4°C, 10 minutes) and aliquoted (2-4 aliquots per patient) and immediately frozen at −80°C. Once all samples were collected, they were sent as a single batch for biomarker assay for the

TABLE 1 The owner-based questionnaire developed by CAWEC

Please indicate how often your dog shows each of the following behaviors			
0	**1**	**2**	**3**
Never	Once a month	Once a week	Almost daily
DISORIENTATION MULTIPLY SCORE BY 2		**SCORE**	
Stares intently where there is nothing visible			
Does not remember its way back home			
Gets stuck behind objects or furniture			
Stays on the wrong side of the door			
Does not respond to certain stimuli to which it used to respond			
Does not give any signal when it wants to go out			
SLEEP-WAKE CYCLES			
Walks during the night (without an obvious reason) when it did not used to do this			
Vocalizes (barks, whines) during the night (without an obvious reason) when it did not used to do this			
SOCIAL INTERACTIONS			
Does not recognize familiar people			
Does not recognize familiar animals			
Shows more signs of fear or aggression toward people and/or other dogs than it used to			
LEARNING AND MEMORY			
Urinates and/or defecates in new (inappropriate) places when it did not use to do this			
Finds it difficult to respond to previously learned commands			
ACTIVITY LEVEL			
Is less active or playful than it used to be			
Shows repetitive behaviors (chases own tail, snaps at "invisible" flies, etc)			
Walks without obvious purpose			
ANXIETY			
Shows more signs of anxiety when separated from its owners than before (main signs of anxiety are shaking, shivering or trembling, excessive salivation, restlessness/agitation/pacing, whining, loss of appetite)			
Interpretation of the score			
0-7	8-40	41-69	
Normal aging	Mild cognitive impairment	Severe cognitive impairment	

Abbreviation: CAWEC, Companion Animal Welfare Education Center.

ABtest service (Araclon Biotech, Zaragoza, Spain). The ABtest service included the measurement of Aβ40 and Aβ42 in plasma and CSF samples using 2 specific ELISA sandwich kits (ABtest 40 and Aβtest 42). The validation and methodologies of ABtest40 and ABtest42, conducted by Araclon Biotech, have been published in independent works.[25] The aforementioned AB tests have been validated in a canine model.[24] Araclon Biotech laboratory staff was blinded to cognitive status and all the other participant characteristics.

The ABtest service included the quantification of Aβ levels in 3 plasma fractions: free Aβ in plasma (FP40 and FP42) measured in undiluted samples, total Aβ in plasma (TP40 and TP42) measured in diluted samples, and Aβ bound to plasma components (CP40 and CP42). The standard Aβ42/Aβ40 ratio in free plasma (FP), TP, and CP (TP42/40, FP42/40, and CP42/40) and a variant based on the indirect markers as it is suggested, were also evaluated.[26] Specifically, the sum of Aβ40 and

Aβ42 in total plasma, which is defined as total β-amyloid in plasma (TPAβ), was calculated.[26] Regarding CSF, Aβ levels were determined following the standard ABtest procedure in properly adapted diluted samples.[12] The ratio of CSF Aβ42/Aβ40 was also determined.

2.3 | Statistical analysis

Both parametric and nonparametric statistical methods were applied for the statistical evaluation of the data. The assumptions of normality and homogeneity of variances for the continuous variables were tested using the Shapiro-Wilk and Levene's test, respectively. In cases where the assumptions of variability, normality, or both of the population's distribution were seriously violated, the Kruskal-Wallis nonparametric test was applied to evaluate group differences.

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Differences between specific groups were evaluated using the non-parametric Wilcoxon rank-sum test (Mann-Whitney *U* test). The Spearman correlation coefficient was estimated to evaluate the correlation between direct and calculated Aβ variables.

Binary logistic regression was applied to investigate whether the level of the biomarkers (predictors), which were converted to dichotomous variables by the median of the pooled population, was associated with an increased likelihood of CDS diagnosis. For this empirical application, aged animals were divided; (1) group 1: CU and group 2: MCI + SCI; (2) group 1: CU and group 2: SCI; (3) Group 1: MCI and group 2: SCI; and (4) group 1: CU and group 2: SCI. The specification chosen for the logistic regression model was based on the statistical significance of the predictors. The association of the biomarkers with dogs' cognitive status assessed through the application of univariable logistic regression models with robust standard errors. The final logistic regression model was built using the biomarkers that presented a strong univariable association with the groups (*P* value < .10) as

predictors. Receiver operating characteristic (ROC) curves were used to assess the accuracy of biomarkers with a significant odds ratio in classifying the presence versus absence of cognitive impairment. The sensitivity and specificity of these blood biomarkers were calculated using the most appropriate cut-off point from their corresponding ROC curve. All analyses were conducted using the statistical software SPSS (v. 25.0) and Stata 11.0. Graphic representations were created using GraphPad Prism 8.0.

3 | RESULTS

3.1 | Reliability and control parameters of the study

The reliability of the results was assessed by evaluating and monitoring the accuracy and reproducibility of each assay. The average data
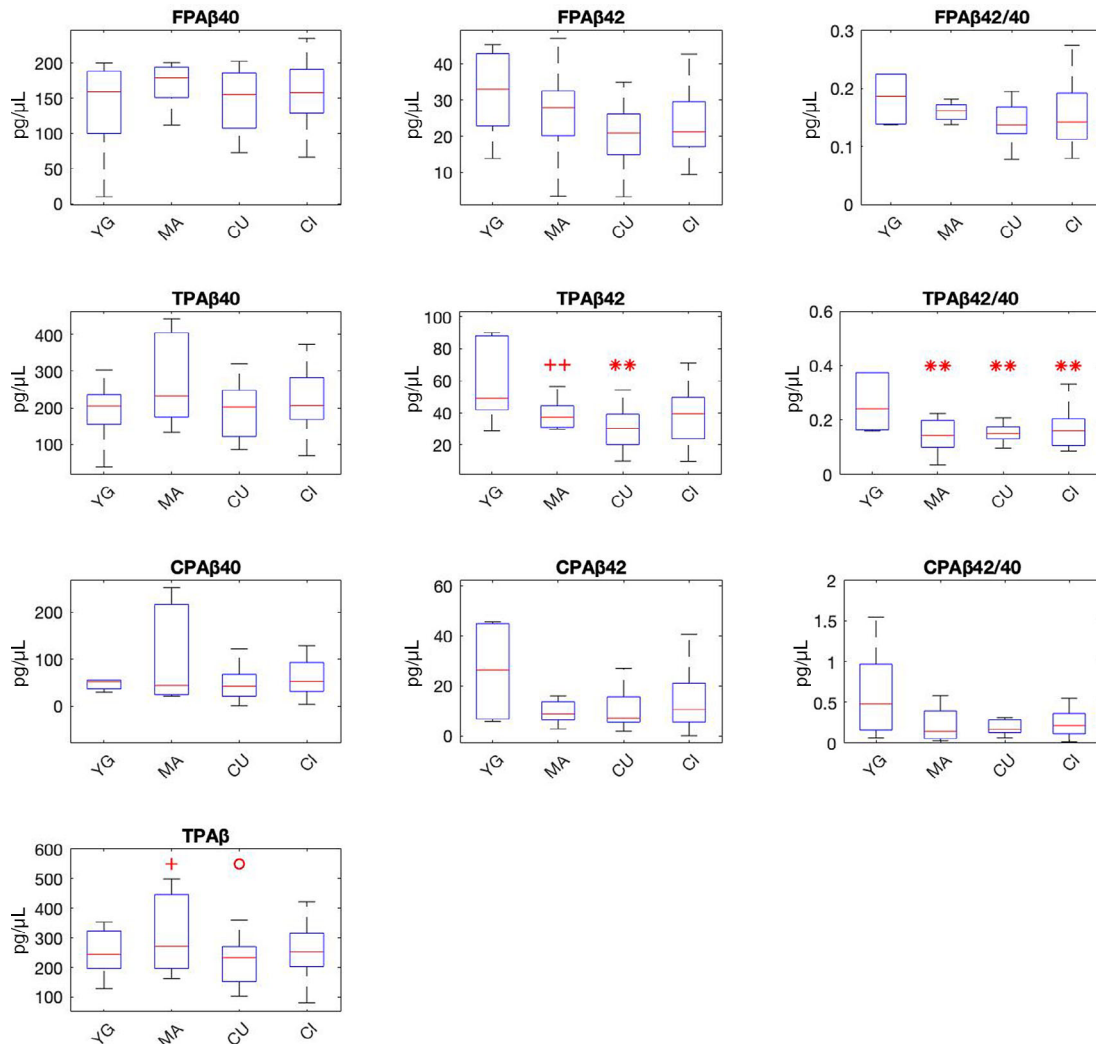

FIGURE 1 Levels (pg/μL) of direct and calculated plasma Aβ markers in each study group. Symbols are shown as exponents in groups where the levels of biomarkers have been exhibited to differ from others, with statistical significance. Namely *, °, + indicate significance concerning young (YG), middle aged (MA), and cognitive unimpaired aged (CU) group, respectively. Significance is indicated with **, °°, ++ for *P* < .05. In accordance, +, *, ° indicate significance *P* < .1

obtained are summarized in Table S1. All these data were within the acceptance criteria for ABtest.

The mean results for the Aβ direct markers in plasma and CSF are presented in Table S2. Based on the pooled values, it was evident that approximately 27% of Aβ40 and 48% of Aβ42 in plasma were bound to plasma components.

3.2 | Comparisons between diagnostic groups

The descriptive statistics of the study are present in Figure 1.

Regarding plasma markers, the YG group had the highest levels of TPAβ42 and TPAβ42/40, followed by the CI aged group. On the contrary, the CU aged group showed the lowest levels of TPAβ42. TPAβ40 levels were significantly higher in the MA group compared to the CU aged group. Significant differences were not detected for other markers.

Moreover, no significant differences were observed between CU and CI aged dogs. However, when the CI dogs were subdivided in MCI and SCI according to their stage of cognitive dysfunction, some direct and indirect markers exhibited significant differences. Specifically, TPAβ42, TPAβ42/40, and CPAβ42 levels were higher in MCI dogs when compared to CU and SCI dogs (Figure 2).

Levels of CSF Aβ40 and Aβ42 were low in the YG group, and higher in the MA group. The levels of Aβ42 were lower in CI aged dogs compared to the CU aged dogs. This difference was significant among CU and MCI dogs. In contrast, Aβ40 levels did not present similar differences between these 2 groups (Table 2).

In the majority of cases, the concentrations of plasma markers were correlated with each other, as seen in Table S3. The levels of CSF Aβ42 were found to be correlated only with the CP fraction of Aβ42 and Aβ40 and the indirect markers, which had not been calculated in all the fragments of canine blood before this study. Conversely, CSF Aβ40 was correlated only with Aβ40 blood markers and
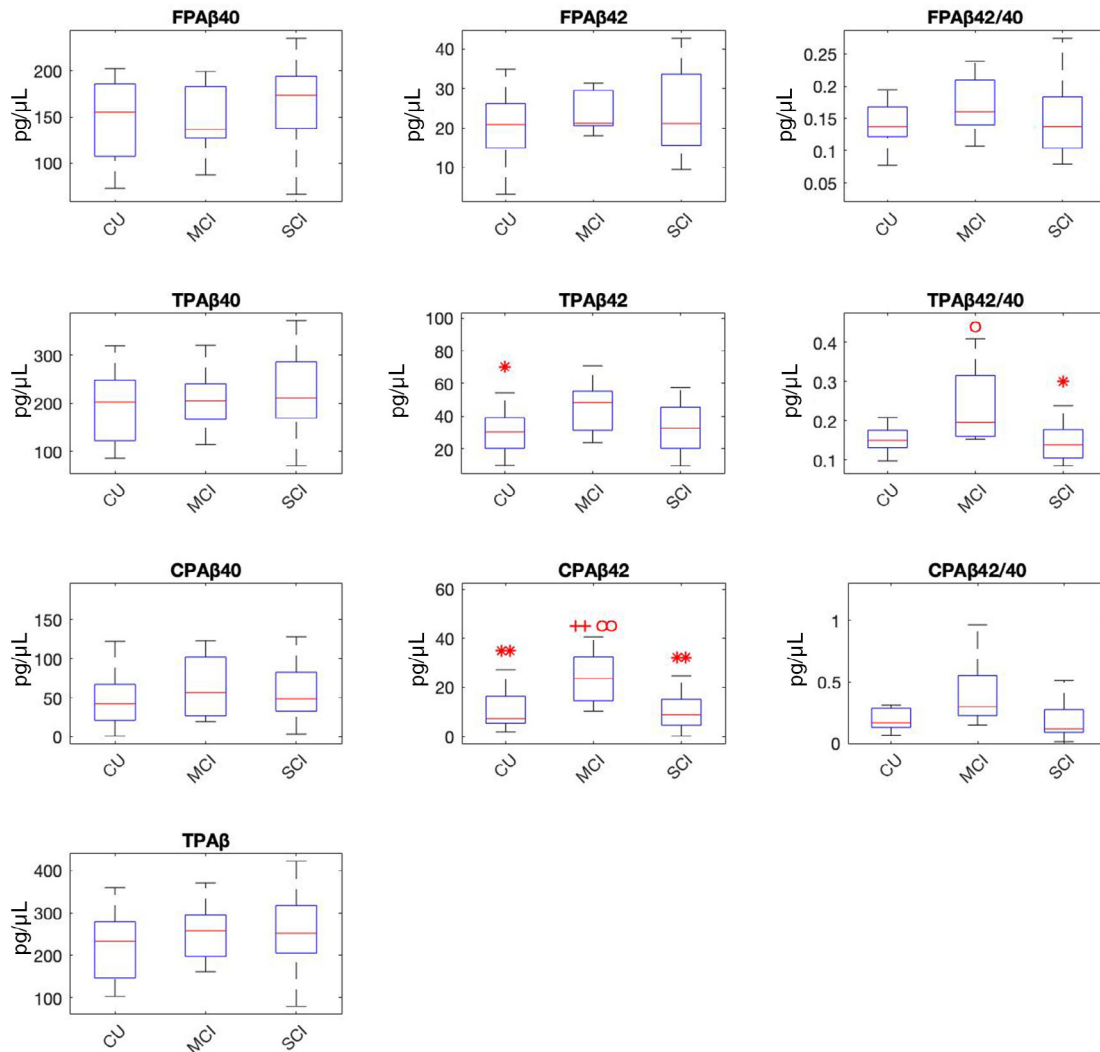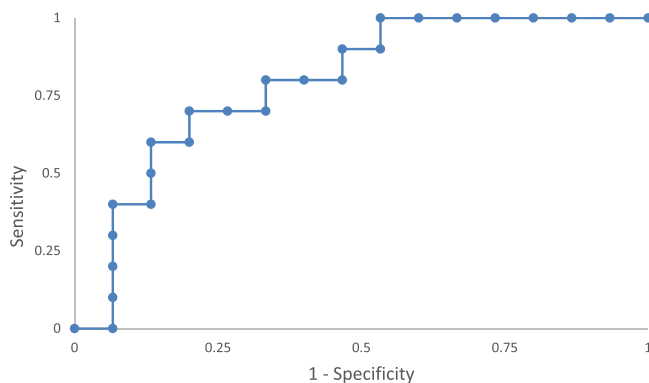

FIGURE 2 Levels (pg/μL) of direct and calculated plasma Aβ markers in the aged dog groups. [+], [*], [°] indicate significance with regard to CU, MCI, and SCI, correspondingly. Significance is indicated with [++], [**], [°°] and [+], [*], [°] for *P* < .05 and .1, respectively. CU, cognitively unimpaired; MCI, mild cognitive impairment; SCI, severe cognitive impairment

TABLE 2 Levels (ng/mL) of CSF Aβ markers in each group of the study

Group	n	CsfAβ40		CsfAβ42		CsfAβ42/40	
		Mean	SD	Mean	SD	Mean	SD
YG	6	6.39	1.02	1.16	0.10	0.18	0.02
MA	8	8.52	3.08	1.34	0.36	0.16	0.02
CU	17	8.47	2.04	1.25	0.28	0.15	0.02
MCI	10	8.13	2.40	1.04	0.32	0.12	0.04
SCI	20	7.55	2.19	1.20	0.15	0.16	0.04

Abbreviations: Aβ, amyloid beta; CSF, cerebrospinal fluid; CU, cognitively unimpaired; MA, middle aged; MCI, mild cognitive impairment; SCI, severe cognitive impairment; YG, young dogs.

TABLE 3 Two cut-off points were selected from the above curve (Figure 1). The first cut-off point (0.151) is referred to as model #1 and the second cut-off point (0.160) is referred to as model #2

	Model #1	Model #2
Cut-off point	0.151	0.160
Sensitivity	90%	80%
Specificity	53.33%	66.67%
Positive predictive value	56.3%	61.5%
Negative predictive value	88.9%	83.3%
Likelihood ratio positive	1.93	2.4
Likelihood ratio negative	0.19	0.3
Accuracy	68%	72%
Diagnostic odd ratios	10.29	8.0

FIGURE 3 Receiver operating characteristic curve of TPAβ42/40 allows discrimination between MCI and CU aged dogs with AUC ≥ 0.79. AUC: area under curve; CU: cognitively unimpaired; MCI: mild cognitive impairment

TPAβ. TPAβ and TPAβ42 demonstrated the strongest correlation with the other markers.

3.3 | Sensitivity and specificity of the markers

An ROC curve analysis of markers that fulfilled the appropriate statistical criteria was performed in order to evaluate their sensitivity and specificity as diagnostic tools. TPAβ42/40 was proven to be suitable for the distinction between MCI and CU aged dogs, with sensitivity and specificity analogous to most diagnostic tests. To date, no established practical cut-off points of Aβ measurements exist in the veterinary field; therefore, we selected to evaluate 2 cut-off points (model #1 and model #2) resulting from the ROC curve with AUC.79 for this biomarker (Figure 3). Breed size did not qualify as a covariate, as it has been shown that breed size does not affect CDS prevalence.[1] Model #1 presented higher sensitivity (90%) but lower specificity (53.33%) and accuracy (68%) when compared to model #2, which presented higher specificity (66.67%) and accuracy

(72%) but lower sensitivity (80%). Thus, we considered that both were accompanied by diagnostic criteria that can be used (even in combination) as tools to characterize the cognitive profiles of animals of interest (Table 3).

4 | DISCUSSION

In our study, we made a combined approach of all Aβ fractions and increased the available database on Aβ metabolism in a clinical setting. TPAβ42 was found increased in MCI dogs compared to CU dogs. Similarly, CPAβ42 was higher in the MCI group compared to the other aged groups. TPAβ42/40 demonstrated value in the differentiation of CU and MCI dogs. As it will be analyzed below, CP fragment is important to be measured. Also, the biomarker's determination could serve in a manifold approach of the disease, emphasizing the TPAβ42/40 potential role. The variance of the concentrations of Aβ fragments in SCI dogs could indicate a feature of the disease.

By measuring all direct and calculated Aβ markers instead of a single peptidepossible bias in Aβ level quantifications was eliminated.[13] Compared to the measurements, which are performed using an earlier version of the current method (ABtest service), the levels of Aβ42 were quite similar, though Aβ40 levels were nearly 3 times higher.[24] We found that 48% of Aβ42 and 27% of Aβ40 represented the fraction of Aβ bound to plasma components (CP). The results of the present indicate that Aβ levels in the blood are higher than those demonstrated by most ELISAs in studies of dog samples.[14,24,26,27] Furthermore, evaluation of CP fraction sheds more light on Aβ profile's in blood, while changes in the distribution of Aβ peptides in plasma could offer more information regarding disease progression.[19,25,28-30] Notably, our findings reinforced this perspective. More specifically, we found that CSF Aβ42 was strongly correlated only with the CPAβ40 and CPAβ42 fractions as well as the calculated marker TPAβ, which also contains the CP fraction. This correlation indicated that the combination of these measurements could be complementary to one another, suggesting that this panel could systematically elucidate the disease staging process. The current challenge involves the detection of CDS in the early phase,

when signs are either absent or mild—ideally when the neuropathological lesions are starting to develop. Correspondingly, in our case, the fact that we demonstrated significant changes in TPAβ42, CPAβ42, and TPAβ42/40 in the early stages of CDS was striking because it suggested that using these markers could provide further answers for clinical and prognostic outlines of the syndrome. Moreover, TPAβ42/40 determination indicated a statistically significant difference between the measurements among MCI and SCI dogs, thereby adding value to animals' cognitive profiles. Therefore, in agreement with previous publications, TPAβ42/40 could function as a substantial marker within current veterinary medicine.[14,24]

Amyloid-beta measurements in SCI dogs could either be affected by confounding factors, or by other nonamyloid brain pathology factors or by the underlying pathology, as mixed pathologies could convolute.[12,26,31-34] Some human studies conclude that an individual's amyloid load accumulates before clinical signs and soon reaches a level beyond which there is either very slow or no further accumulation despite the clinical advancement of the disease.[26,35] Based on this conclusion, our results led us to support the notion that lower Aβ measurements among SCI compared to MCI dogs, the lack of significant difference between CU and SCI aged dogs, and the variability of the Aβ levels of SCI dogs could be explained. This variability of Aβ measurements could reflect an actual characteristic of this disease. Numerous human studies on late-onset AD report a relationship between elevated plasma Aβ peptide levels and the disease. These studies suggest that increased Aβ blood levels represent an early event preceding the symptomatology and denote a higher chance of developing the disease.[31,36-44] Nevertheless, contradictory studies report no association between Aβ plasma levels and AD development.[45-51]

In our study, clinical cognitive aging classification was accomplished using an owner-based questionnaire. Notably, CDS scales are the primary diagnostic tool for veterinarians. A wealth of research has been performed for the development of a questionnaire to classify the cognitive status of aged dogs in a reliable and sensitive manner.[4,7,24,52-56] However, since questionnaires rely heavily on owners' judgment and because the design and scoring methods vary, this weakens their contribution to the detection of early and subtle changes in the cognitive function of aged dogs.[9] Recently, it is suggested to measure the cognitive aging in pet dogs with a group of tests evaluating different cognitive functions, including human-animal interaction.[9,57] This is called the Vienna Canine Cognitive Battery test, which can be performed within 1 to 2 hours, thus making it practical in general veterinary practices.[9] Our results encourage this combination strategy, which suggests that the sensitivity of TPAβ42/40 markers highlighted in this study can increase the precision of cognitive assays under the current rationale for CDS clinical diagnosis.

In human medicine, CSF biomarkers—including Aβ peptides and Tau protein—are considered standardized and valuable means for the early detection of AD, presenting a sensitivity of 95% and specificity of 87%.[12,51,58-60] In dogs, CSF Aβ42 decreases with age, while Aβ40 remains stable.[20] Moreover, CSF Aβ42 and Aβ42/Aβ40 are able to predict both Aβ42 and Aβ40 burden in the brain.[20] In our study, dogs

4-8 years old had the highest concentrations of CSF Aβ42 (1.34 ± 0.36 ng/mL) compared to CI aged group (1.14 ± 0.23 ng/mL, $P = .17$) and young group (1.16 ± 0.10 ng/mL, $P = .39$). Furthermore, CU aged group had higher concentrations of Aβ42 (1.25 ± 0.28 ng/mL) than MCI aged group (1.04 ± 0.32 ng/mL, $P = .09$). Conversely, Aβ40 levels were similar. These results could reflect the increased deposition of Aβ42 detected in CI dogs as well as the low deposition of Aβ40 found in brain tissue of aged dogs.

The present study led us to consider that the quantification of Aβ biomarkers, ideally in both CSF and plasma, should be incorporated in the diagnostic protocol for CDS. This approach would be useful in many ways. First, TPAβ42/40 could generate a new recruitment strategy using the cut-off values of models #1 and #2 for efficient preselection in the population of interest, as it is applied in human medicine.[10] Since this step concerns the earliest time of the signs, TPAβ42/40 determination in combination with the other clinical methods used (ie, questionnaires and battery test) could facilitate a more precise description of patient profiles at each stage of the disease. This implies that it could also be used as a tool in studies concerning the evaluation of different means of CDS clinical diagnosis. Second, the significant difference of plasma CP Aβ distribution in dogs with MCI compared with the other groups denotes that measuring CP fragments could contribute to the more complete identification of baseline characteristics at the early stages of the syndrome. Third, the correlation of CSF measurements with plasma markers (especially with the CP fragment) indicates that determining a combination of CSF and plasma markers—instead of a singular measurement—could act in a comprehensive manner to establish the complex regulation of Aβ peptides in cognitively unimpaired and impaired dogs. While further research is required to clearly establish the role of Aβ as a diagnostic tool for CDS, the reported results are encouraging. For this reason, we suggest the use of a panel including TPAβ42/40, TPAβ42, CPAβ42, CPAβ42/40, and CSFAβ42 as very promising indicator for the early diagnosis, prognosis, and accurate definition of each stage of cognitive function changes and the preventive treatment of CDS.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by the Ethical Committee of the Veterinary School, Aristotle University of Thessaloniki, Greece (72/25-10-16).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.