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Plasma Amyloid Beta Concentrations in Aged and Cognitively Impaired Pet Dogs

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Abstract

Longevity-associated neurological disorders have been observed across human and canine aging populations. Alzheimer's disease (AD) and canine cognitive dysfunction syndrome (CDS) represent comparable diseases affecting both species as they age. Translational diagnostic and therapeutic research is needed for these incurable diseases. The amyloid β (Aβ) peptide family are AD-associated peptides with identical amino acid sequences between dogs and humans. Plasma Aβ42 concentration increases with age and decreases with AD in humans, and cerebrospinal fluid (CSF) concentration decreases in AD and correlates inversely with the amyloid load within the brain. Similarly, CSF Aβ42 concentrations decrease in dogs with CDS but there is limited and conflicting information on plasma Aβ42 concentrations in aging dogs and dogs with CDS. We measured plasma concentrations of Aβ42 and Aβ40 with an ultrasensitive single-molecule array assay (*SIMOA*) in a population of healthy aging dogs of different life stages (n = 36) and dogs affected with CDS (n = 11). In addition, the ratio of Aβ42/β40 was calculated. The mean plasma concentrations of Aβ42 and Aβ40 increased significantly with age (r^2 = 0.27, $p = 0.001$; and r^2 = 0.42, $p < 0.001$, respectively) and with life stage: puppy/junior group (0.43–2 years): 1.23 ± 0.95 and 38.26 ± 49.43 pg/mL; adult/mature group (2.1–9 years): 10.99 ± 5.45 and 131.05 ± 80.17 pg/mL; geriatric/senior group (9.3–14.5 years): 18.65 ± 16.65 and 192.88 ± 146.38 pg/mL, respectively. Concentrations of Aβ42 and Aβ40 in dogs with CDS (11.0–15.6 years) were significantly lower than age-matched healthy dogs at 11.61 ± 6.39 and 150.23 ± 98.2 pg/mL ($p = 0.0048$ and $p = 0.001$), respectively. Our findings suggest the dynamics of canine plasma amyloid concentrations are analogous to that found in aging humans with and without AD.

Keywords Canine · Cognitive dysfunction · Alzheimer's disease · CDS · Amyloid beta 42 · Amyloid beta 40

Introduction

Alzheimer's disease (AD) and canine cognitive dysfunction syndrome (CDS) are comparable diseases affecting human and canine species as they age [1]. An AD epidemic is evolving across the globe and has been connected to an increased average life expectancy [2]. Demographic studies have shown that aged pet dogs also suffer from age-associated neurodegenerative processes [3, 4]. A core pathophysiological feature shared across human and canine species is spontaneously occurring amyloid β (Aβ) plaque deposition within the extracellular space in the brain, along with Aβ-induced neuronal and glial proteotoxicity [5]. The four-stage distribution of Aβ deposits within the cortical gray matter that has been described in people has been also identified in dogs using immunohistochemistry [6]. Importantly, the amino acid sequence of Aβ is identical between humans and dogs [7]. Brain pathology correlates with the severity of cognitive impairment in both species as described in several studies [8–12]. In humans with

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AD, there is a decrease in concentration of Aβ within the cerebrospinal fluid (CSF) which negatively correlate with amyloid load in the brain [13, 14]. Similarly, in beagle dogs with CDS, the concentrations of Aβ oligomers in the CSF are inversely correlated with Aβ deposition when assessed with enzyme-linked immunosorbent assay (ELISA) [15] or electrochemiluminescence detection technology [16].

Advances in minimally invasive blood-based biomarkers have enabled early screening for non-AD individuals at greatest risk for the development of AD. These biomarkers can be used as sensitive outcome measures in clinical trials and to analyze the therapeutic effect of experimental drugs [17]. Proteins such as Aβ42 and Aβ40 are a component of AD pathology and can be detected in blood. However, ultrasensitive technologies are required for their reliable measurement [18]. Single-molecule array assay (*SIMOA*) technology represents a novel, highly sensitive platform that can detect thousands of single molecules simultaneously. While traditional ELISA systems require a high number of enzyme labels to generate reader-detectable signals, *SIMOA* utilizes femtoliter-sized reaction chambers that can isolate and detect single enzyme molecules. In humans, plasma Aβ oligomer concentrations measured with SIMOA decrease several years before a clinical diagnosis of AD [19]. In addition, low plasma concentrations of Aβ42 combined with high plasma concentrations of neurofilament light chain (NfL) are associated with all-cause and AD dementia [19]. These findings confirm that both biomarkers can be used as minimally invasive tools to predict AD onset in non-AD population. We have recently shown that plasma NfL can be successfully measured in a population of pet dogs with the single-molecule array assay (SIMOA) technology [20]. In this study, we have used the same SIMOA technology to explore plasma concentrations of the amyloid oligomers amyloid-β42 (Aβ42) and amyloid-β40 (Aβ40) in healthy dogs at different life stages and in elderly dogs affected with CDS. In addition, we evaluated the dynamics of the Aβ42/Aβ40 ratio. Our findings confirmed that plasma concentrations of Aβ42 and Aβ40 can be successfully measured using SIMOA in pet dogs and that their concentrations increase with age and decrease with the presence of CDS. Our data mirrors recent human literature and suggests that future, large-cohort studies assessing plasma Aβ with SIMOA may provide an important means of monitoring disease state and response to therapy, enhancing the translational potential of this naturally occurring model.

Methods

Animals

This study included 47 dogs recruited from the staff of the NC State College of Veterinary Medicine. There were 36 healthy dogs and 11 suffering from CDS. Dogs were stratified using life stage categories based on the American Animal Hospital Association (AAHA) canine life stage guidelines [21] combined with breed lifespan by the American Kennel Club (AKC). Inclusion criteria included a normal physical examination, normal vision, intact hearing, and the ability to walk independently. Dogs were excluded if they had focal neurological deficits on neurological examination indicating an underlying neurological condition distinct from age-related dementia, if they had an active neurological condition such as epilepsy that could alter plasma biomarker concentrations, or if they were being treated with behavior-modifying medications such as serotonin antagonists/reuptake inhibitors.

All dogs underwent physical, orthopedic, and neurological examinations followed by a blood draw. Cognitive status was established using the Canine Dementia Scale (CADES (Suppl Fig. 1) [22]. This scale is completed by owners and assigns a score based on owner assessment of 17 items, grouped into spatial orientation, social interactions, sleep-wake cycles, and house soiling. Cognitive impairment was then classified for each dog as normal, mild, moderate, or severe. All owners were provided with details of the study, were given the opportunity to ask questions, and signed an informed consent. All procedures were performed in accordance with the North Carolina State University Institutional Animal Care and Use Committee. Data gathered on the dogs included age, breed, sex, and health status. Table 1 summarizes the demographic and clinical information for participants grouped according to life stage and disease status.

Measurement of Plasma Aβ42 and Aβ40 Concentrations

Blood samples were taken into EDTA tubes and centrifuged at 2000×*g* at 4 °C for 8 min within 2 h of collection. Plasma supernatant was collected, divided into aliquots, and frozen at − 80 °C until further use. Samples were thawed on ice before analysis and the concentration of plasma Aβ42 and Aβ40 was measured using the Single Molecule Array Assay kit (AB40 and AB42, Quanterix, Lexington, MA), following the manufacturer's instructions. Intra-assay coefficient of variation has been described to vary between 0.1 and 8%, and inter-assay coefficient of variation between 2 and 8%. The limit of detection for Aβ42 assay was 0.034 pg/mL (range 0.014–0.052 pg/mL) and for Aβ40 assay was 0.17 pg/mL (range 0.092–0.28 pg/mL).

Statistical Analysis

Summary data were prepared on age, weight, sex, breed, CADES score, and Aβ42, Aβ40, and Aβ42/40 ratio with dogs grouped according to life stage. In the first analysis, the relationship between age, weight, and sex on Aβ42, Aβ40, and Aβ42/40 was examined in healthy dogs using linear

Table 1 Demographic features of study participants. Means (SD) have been used to present plasma concentrations of Aβ42 and Aβ40 and the ratio of Aβ42/40. *CDS*, cognitive dysfunction syndrome; *AAHA*, American Animal Hospital Association; *CADES*, Canine Dementia Scale; *plasma Aβ42*, amyloid beta 42; *plasma Aβ40*, amyloid beta 40; *plasma Aβ42/40*, ratio of Aβ42 and Aβ40; *SD*, standard deviation

	Number of dogs (*n*)	Life stage (AAHA)	Age (years)	Dementia score (CADES)	Plasma Aβ42 (pg/mL) (mean ± SD)	Plasma Aβ40 (pg/mL) (mean ± SD)	Plasma Aβ42/40 (pg/mL) (mean ± SD)
Healthy controls	*n* = 12	Puppy/junior	0.40–2	Normal	1.23 ± 0.95	38.29 ± 49.43	0.19 ± 0.15
	n = 14	Adult/mature	2.1–9	Normal	10.99 ± 5.45	131.05 ± 80.17	0.11 ± 0.03
	n = 10	Senior/geriatric	9.3–14.5	Normal	18.65 ± 16.65	192.88 ± 146.38	0.10 ± 0.05
Cognitive dysfunction syndrome (CDS)	*n* = 11	Senior/geriatric	11.0–15.6	Mild, *n* = 4	13.78 ± 8.85	198.51 ± 94.52	0.08 ± 0.05
				Moderate, *n* = 2	11.34 and 7.19	94.26 and 47.84	0.12 and 0.15
				Severe, *n* = 5	10.87 ± 5.78	143.26 ± 107.76	0.10 ± 0.05

regression. Plasma Aβ42 and Aβ40 concentrations and the ratio of Aβ42/40 concentration were compared between the life stage groups of healthy dogs using the Wilcoxon test for each pair. The relationship between CDS and plasma Aβ42 and Aβ40 concentrations and the ratio of Aβ42/40 concentrations was examined in two different ways. Firstly, a model was built to examine the effect of disease status (healthy vs CDS) on plasma Aβ42, Aβ40, and Aβ42/40 concentrations with age as a covariate. Finally, logistic regression was used to examine the relationship between CADES score and Aβ42, Aβ40, and Aβ42/40 concentrations in senior/geriatric dogs only, both with and without CDS. Statistical analyses were performed using JMP14 (SAS Institute, Cary, NC). All reported *p* values were considered to be statistically significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$. Data are presented as mean ± standard deviation.

Results

Clinical Characteristics

Forty-seven pet dogs (26 males and 21 females) of various breeds (Beagle *n* = 8, Boxer *n* = 3, Border Collie *n* = 4, Cairn Terrier *n* = 2, German Shepherd dog *n* = 3, Golden Retriever *n* = 1, Hound *n* = 1, Jack Russell Terrier *n* = 2, Labrador Retriever *n* = 7, Mix Breed *n* = 12, Pembroke Welsh Corgi *n* = 2, Rottweiler *n* = 2) were enrolled in this study.

Of these, 36 were healthy and 11 were diagnosed with CDS. The ages of dogs ranged from 0.40 to 15.6 years. The population of healthy pet dogs included puppy/junior dogs (0.40–2 years, *n* = 12), adult/mature dogs (2.1–9 years, *n* = 14), and senior/geriatric dogs (9.3–14.5 years, *n* = 10). All dogs with CDS were senior/geriatric (11.0–15.6 years). Demographic features of study participants and CADES dementia scores are presented in Table 1.

Plasma Aβ42 and Aβ40 Concentrations in 36 Healthy Pet Dogs Increase with Age

Plasma concentrations of Aβ42 and Aβ40 and the Aβ42/β40 ratio are shown in Table 1. Plasma Aβ42 concentrations increased with age ($r^2 = 0.27$, $p = 0.001$) (Fig. 1a) and were not affected by body weight ($r^2 = 0.038$, $p = 0.25$) and/or sex ($r^2 = 0.14$, $p = 0.07$). Similarly, plasma Aβ40 concentrations increased with age ($r^2 = 0.42$, $p < 0.001$) (Fig. 1b) and were not affected by body weight ($r^2 = 0.05$, $p = 0.17$) and/or sex ($r^2 = 0.16$, $p = 0.055$). The ratio of Aβ42/β40 decreased with age ($r^2 = 0.15$, $p = 0.02$) and was not affected by body weight ($r^2 = 0.02$, $p = 0.41$) nor sex ($r^2 = 0.01$, $p = 0.49$) in the population of healthy pet dogs (Fig. 1c). With dogs grouped according to life stage, the plasma concentrations of both peptides were significantly different between each group as illustrated in Fig. 2. Both Aβ42 and Aβ40 increase in concentration with increasing maturity of life stage, while there was a decrease in the Aβ42/β40 ratio.

Plasma Aβ42 and Aβ40 Concentrations Are Lower in Dogs with CDS and Correlate Negatively with the CADES Score

Plasma concentrations of Aβ42 and Aβ40 and the Aβ42/β40 ratio in dogs with CDS are shown in Table 1. When concentrations of Aβ42 and Aβ40 were compared between 11 dogs affected with CDS and 10 healthy senior/geriatric dogs using multivariate analysis with age as a covariate, dogs with CDS exhibited lower concentrations of Aβ42 and Aβ40 than healthy dogs ($r^2 = 0.46$, $p = 0.0048$; and $r^2 = 0.61$, $p = 0.001$, respectively) (Fig. 3a and b). The ratio of Aβ42/β40 did not significantly differ between the CDS and healthy senior/geriatric group ($r^2 = 0.1$, $p = 0.4$) (Fig. 3c). A negative correlation was found between plasma concentrations of Aβ42 and Aβ40 and

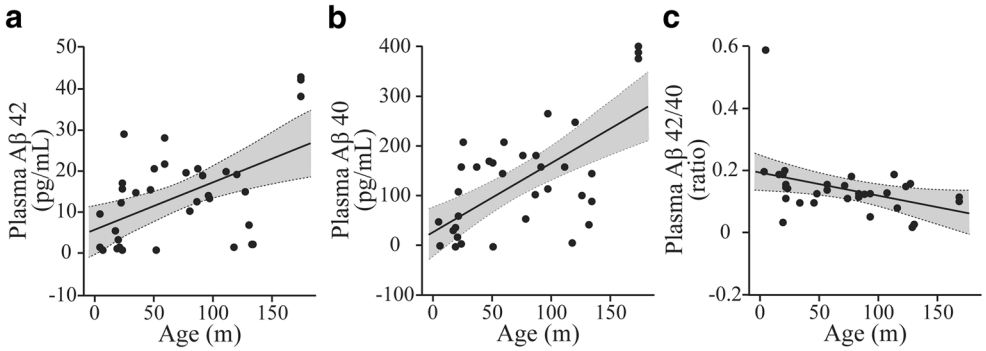

Fig. 1 Plasma amyloid beta 42 (Aβ42) and amyloid beta 40 (Aβ40) concentrations increase with age in the population of healthy pet dogs (a, b). The ratio of Aβ42/40 decreases with age in the same population (c). A multiple linear regression model was fitted using plasma Aβ42, Aβ40, and Aβ42/40 concentrations as response variables and age, sex, and body weight as dependent variables. There was a positive correlation between age and plasma Aβ42 and Aβ40 ($r^2 = 0.27$, $p = 0.001$; and $r^2 = 0.42$, $p < 0.001$, respectively). There was a negative correlation between age and the ratio of Aβ42/40 ($r^2 = 0.15$, $p = 0.02$)

CADES scores in senior and geriatric dogs (including dogs with CDS) ($r^2 = 0.34$, $p = 0.02$; and $r^2 = 0.59$, $p = 0.002$, respectively) (Fig. 4a and b), but there was no relationship between the plasma Aβ42/Aβ40 ratio and CADES score ($r^2 = 0.12$, $p = 0.3$) (Fig. 4c).

Discussion

In this study, we report that in healthy pet dogs, plasma Aβ42 and Aβ40 concentrations increase in an age-dependent manner and are not affected by body weight or sex. In addition, we show that CDS-affected pet dogs have significantly reduced plasma concentrations of Aβ42 and Aβ40, when compared with cognitively intact, life stage–matched healthy individuals. The ratio of Aβ42/β40 decreased with age in a healthy population of pet dogs; however, no statistically significant difference was noted between CDS-affected and healthy senior/geriatric study participants for this ratio.

Both humans and dogs suffer from similar age-related neurodegenerative diseases, such as AD and CDS [1]. Importantly, both diseases occur spontaneously and both species are exposed to the same environmental conditions and potential neurotoxins [23]. Both also share several neuropathological hallmarks including extracellular deposition of amyloid beta plaques, cerebral amyloid angiopathy, frontal and temporal cortical atrophy, and neuro-axonal loss [5]. Intracellular accumulation of hyperphosphorylated tau protein has been also observed across both species, but the formulation of neurofibrillary tangles (NFTs) composed of this protein has long been recognized as a human-specific feature [24–26]. Recently, it has been shown that dogs affected with CDS share 99% sequence homology for tau microtubular binding domains (MBD) and that they can develop argyrophilic tau fibrillary tangles within the cortex and hippocampus [27, 28].

Numerous studies have been performed to identify relevant plasma biomarkers in humans with AD; however, the number of studies in CDS-affected pet dogs is limited [29]. The

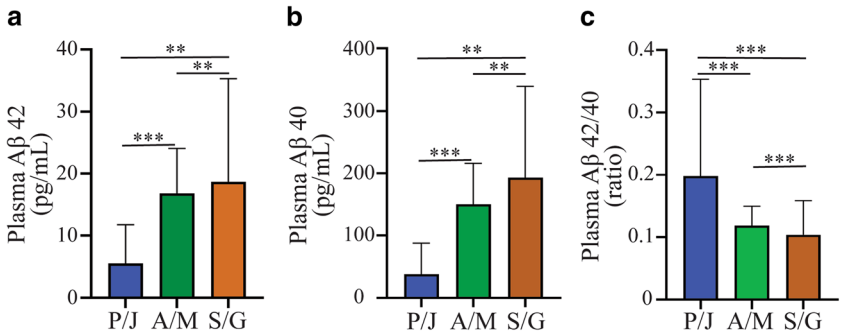

Fig. 2 Plasma amyloid beta 42 (Aβ42) and amyloid beta 40 (Aβ40) concentrations increased in an age-dependent manner in the population of healthy pet dogs stratified into different life stage groups (a and b). The ratio of amyloid beta 42/amyloid beta 40 (Aβ42/40) decreased with age (c) between the life stage group. P/J, puppy/junior ($n = 12$); A/M, adult/mature ($n = 14$); S/G, senior/geriatric ($n = 10$). There were statistically significant differences between the groups and when each group was compared pairwise with the Wilcoxon test: **a** P/J versus A/M ($p < 0.0001$), P/J versus S/G ($p = 0.006$), A/M versus S/G ($p = 0.006$); **b** P/J versus A/M ($p < 0.0001$), P/J versus S/G ($p = 0.002$), A/M versus S/G ($p = 0.002$); **c** P/J versus A/M ($p < 0.0001$), P/J versus S/G ($p = 0.0002$), A/M versus S/G ($p = 0.0002$). ($p < 0.01$*, $p < 0.001$**, and $p < 0.0001$***)

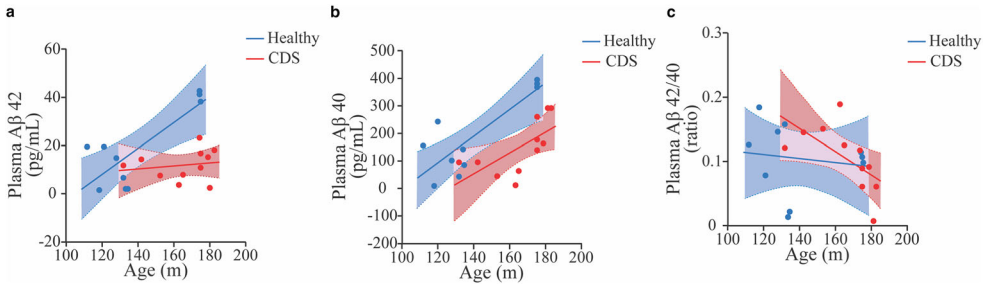

Fig. 3 The relationship between age and plasma amyloid beta 42 (Aβ42) (a), amyloid beta 40 (Aβ40) (b), and ratio of amyloid beta 42 and amyloid beta 40 (Aβ42/40) (c) concentration in a population of healthy senior/geriatric dogs (*n = 10*) and senior/geriatric dogs affected with CDS (*n = 11*). There was a significant difference between these groups for Aβ42 and Aβ40 when evaluated using logistic regression: a $r^2 = 0.46$, $p = 0.0048$; b $r^2 = 0.61$, $p = 0.001$. Analysis of the Aβ42/40 ratio did not show significance: c $r^2 = 0.1$, $p = 0.4$

identification of clinically relevant, non-invasive plasma biomarkers for CDS would not only supplement current veterinary knowledge, but also facilitate bi-directional flow of translational research between the two species. Amyloid beta peptides are the products of enzymatic cleavage of amyloid precursor protein performed by β and γ-secretases as well as β-site amyloid precursor protein-cleaving enzyme 1 [30]. The 42 amino-acid isoform of Aβ peptide is the most commonly found insoluble deposit in AD- and CDS-affected brains [31, 32]. Several previous studies have indicated that CSF concentration of Aβ42 decreases in humans with AD and in dogs with CDS and that decreased concentrations inversely correlate with amyloid beta depositions within the brain parenchyma [13–15]. The concentration of CSF amyloid beta 40 (Aβ40) has been also evaluated in both species; however, its correlation with the presence of AD dementia is less clear [33]. By contrast, several human studies reported that the ratio of Aβ42/40 had higher diagnostic potential than Aβ42 alone [34]. A number of positive emission tomography (PET)–based studies using different Aβ PET tracers have been performed in both dogs and humans and revealed correlations between Aβ42 in CSF and amyloid PET [35, 36]. Using PET imaging, the concentration of Aβ42 in CSF Aβ42 decreases before parenchymal amyloid beta is detectable within the brain suggesting that it is more sensitive marker in the early stages of AD [37].

The CSF contains molecules of great diagnostic and prognostic value, but sampling CSF is invasive and in dogs requires general anesthesia. Unfortunately, only a fraction of molecules detected in the CSF reach the vascular system [38], but high sensitivity technologies have enabled their measurement in peripheral blood, significantly advancing the neurodegenerative field. To date, there have been two contradictory studies that assessed plasma concentrations of Aβ42 and Aβ40 in healthy dogs and dogs with CDS. Both studies used enzyme-linked immunosorbent assay (ELISA) to establish plasma concentrations and both included different approaches to perform cognitive status evaluation [21, 39]. A study by Gonzalez-Martinez et al. [39] was performed on a cohort of eighty-eight pet dogs stratified into different age categories. The age of dogs classified as "young" ranged between 1 and 4 years, for "middle age" between 5 and 8 years, and for "old unimpaired" and "old impaired" was greater than or equal to 9 years. All dogs recruited into the study were small to medium size. This study found that younger dogs had higher plasma Aβ42 and Aβ40 concentrations than healthy aged dogs and that dogs suffering from only mild cognitive impairment had high concentrations of Aβ42 and a high Aβ42/40 ratio. Dogs diagnosed with severe cognitive impairment exhibited comparable values to control individuals. Plasma Aβ40 did not correlate with presence nor severity of CDS. It is notable that although the concentration of plasma Aβ40 was higher than Aβ42, both

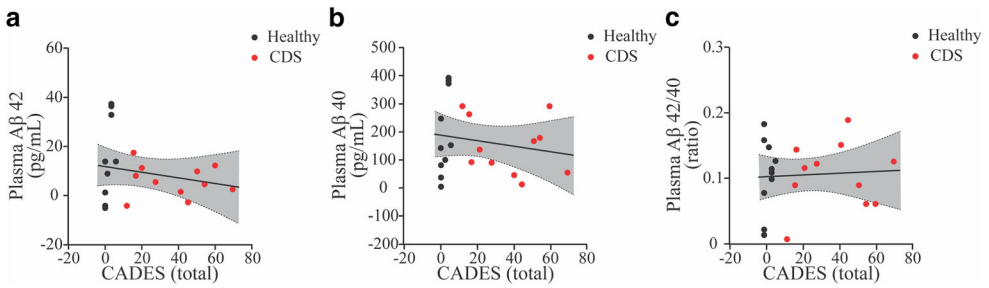

Fig. 4 The relationship between plasma amyloid beta 42 (Aβ42), amyloid beta 40 (Aβ40), and the ratio of amyloid beta 42/amyloid beta 40 (Aβ42/40) concentration and Canine Dementia Scale (CADES) score in healthy senior/geriatric dogs (*n* = 10) and senior/geriatric dogs with CCD (*n* = 11). Plasma Aβ42 and Aβ40 concentrations are associated with the CADES score when evaluated using logistic regression: **a** Aβ42 $r^2 = 0.34$, $p = 0.02$; Aβ40 $r^2 = 0.59$, $p = 002$. The ratio of Aβ42/40 did not significantly correlate with *CADES* score: $r^2 = 0.12$, $p = 0.3$

were within the same level of magnitude. Similar to our study, the majority of human studies report plasma concentrations for Aβ40 10–20 times higher than for Aβ42 within the same individuals. The study by Schutt et al. [23] included fifteen pet dogs of different breeds and age (between 9 and 15 years old) and evaluated the correlation between plasma concentrations and brain deposition of amyloid beta via immunohistochemistry and *ELISA*. Specific plasma concentrations were not reported; however, plasma Aβ42 concentration correlated with maturation stage of Aβ plaques. No significant correlations existed between plasma Aβ42 and cognitive status or with soluble or insoluble fractions of Aβ42 quantified within the prefrontal cortex. It is unclear why these studies' findings differed from ours, but possible reasons include the differing canine populations, Aβ42 and 40 assays, and means of evaluating cognitive status. Our study used the CADES scale to establish cognitive status, while these studies used either the Canine Cognitive Dysfunction Rating (CCDR) scale [40], which appears to be less sensitive to mild cognitive impairment than the CADES scale [20], or a set of scales which have not been compared directly with the CADES scale [9].

In the current study, we used the *SIMOA* assay to measure plasma concentrations of Aβ42 and Aβ40 and established the level of dementia using the validated CADES scale. By stratifying dogs into different life stages, we were able to account for breed-related differences in longevity and to correlate the life stages to human life stages.

Our study had some limitations, including a relatively low number of dogs affected with CDS. It was a cross-sectional rather than a longitudinal study, and longitudinal data would add greatly to the strength of observations made. Dogs were diagnosed with CDS based on physical and neurological examination to exclude metabolic and focal neurologic processes, as well as consistent history. The addition of brain MRI and CSF analysis and ultimately necropsy confirmation would enhance the study.

Our findings that plasma amyloid beta concentrations increase in an age-dependent manner in a population of healthy individuals and decrease with the presence of CDS mirror findings in humans. Importantly, our results provide a strong rationale for large-scale studies in companion dogs which may contribute to acceleration of current translational progress in AD studies.

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Author Contributions W.K.P. and N J.O. conceived and designed the study, analyzed data, and wrote the manuscript. W.K.P performed experiments. W.K.P and R.D.D performed sample processing and testing. N.J.O., D.M. M., M.E. G, and F.M. M and provided critical feedback and oversaw the research program. All authors listed reviewed the manuscript and provided feedback with revisions.

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Compliance with Ethical Standards All procedures were performed in accordance with the North Carolina State University Institutional Animal Care and Use Committee.

Conflict of Interest The authors declare that they have no conflict of interest.

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