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Cognitive Function, Progression of Age-related Behavioral Changes, Biomarkers, and Survival in Dogs More Than 8 Years Old

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Background: Canine cognitive dysfunction (CCD) is an age-dependent neurodegenerative condition dominated by changes in behavioral patterns. Cohort studies investigating cognitive status in dogs are lacking.

Objectives: To investigate cognitive function, progression of age-related behavioral changes, survival, and possible biomarkers of CCD in aged dogs.

Animals: Fifty-one dogs >8 years old; 21 with no cognitive deficits, 17 with mild cognitive impairments (MCI) and 13 with CCD.

Methods: Longitudinal study. Recruitment period of 12 months and an observational period of 24 months including a baseline and 3 planned subsequent assessments. Cognitive status was determined using validated questionnaires. Plasma Aβ-peptides were quantified using commercial ELISA assays and cytokines by a validated immunoassay.

Results: Signs characterizing dogs with CCD were aimless wandering, staring into space, avoid getting patted, difficulty finding dropped food and anxiety. Thirty-three percent of dogs with a normal cognitive status progressed to MCI and 22% classified as MCI progressed to CCD during the study period. For 6 dogs diagnosed with CCD, signs of cognitive dysfunction increased with time. A diagnosis of CCD did not affect survival. The level of plasma $A\beta_{42}$ was significantly increased ($P < .05$) in the CCD group (92.8 ± 24.0 pg/mL) compared to the MCI (77.0 ± 12.3 pg/mL) and normal group (74.9 ± 10.0 pg/mL), but no significant differences in concentrations of systemic inflammatory markers were detected.

Conclusions: Canine cognitive dysfunction is a progressive disorder with an individual variability in the rate of cognitive decline and clinical signs. Plasma $A\beta_{42}$ seems to be an interesting plasma biomarker of CCD.

Key words: Amyloid-beta; Canine; Dementia; Geriatric; Longitudinal.

Senior dogs (those aged > 8 years) spontaneously can develop neurodegenerative cerebral changes and associated impairment of cognitive functions. A specific clinical syndrome characterized by cognitive changes which are not normal for age and cannot be explained by other medical conditions occurs in dogs more than 8 years of age[1–3] and shares multiple similarities to human dementia of the Alzheimer's type.[4–8] The syndrome is referred to as canine cognitive dysfunction (CCD),[2,9] cognitive dysfunction syndrome,[1] and canine counterpart of senile dementia of the Alzheimer's type.[10] The term CCD shall be used in this article.

The prevalence of CCD ranges from 14 to 35% in companion dogs more than 8 years of age, and the risk of developing CCD increases exponentially with increasing age.[11–14] Changes in behavior and daily routines are considered the most important clinical markers of cognitive dysfunction in aged dogs. Therefore, the diagnosis of CCD is primarily driven by owner-based questionnaires and clinical rating scales addressing behavioral alterations within the categories disorientation, social interaction, sleep-wake cycle disturbances, house-soiling, and changes in activity.[1,3,9,15,16] Furthermore, signs of fear and anxiety that have not been present at a younger age are common in dogs with CCD.[1,2] Although developed from different designs and strategies, the dementia score from 3 of the existing CCD screening questionnaires correlated well.[10] The result obtained from CCD questionnaires must, however, always be supported by a thorough clinical evaluation in order to exclude systemic or primary behavioral conditions that could possibly be causing the signs displayed by the affected dog.[1]

The natural history of cognitive dysfunction in senior dogs is sparsely documented.[2,17] It is therefore desirable to gain more information regarding the clinical phenotype, progression, and prognosis of CCD. For this purpose, epidemiological longitudinal studies are needed.

Abbreviations:

AD	Alzheimer's disease
Aβ	amyloid-beta
CCD	canine cognitive dysfunction
CCDR	canine cognitive dysfunction rating scale
CSF	cerebrospinal fluid analysis
CT	computed tomography
HRP	horse-radish peroxidase
MCI	mild cognitive impairment
MRI	magnetic resonance imaging
MSD	Meso Scale Discovery®
OP	optical density
SD	standard deviations

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Another area of interest is to search for potential biomarkers, which in the future might help clinicians to identify dogs suffering from CCD and prompt early supportive initiatives.

The aim of the present study was to provide longitudinal information of cognitive function, progression of age-related behavioral changes and survival in a cohort of dogs more than 8 years old with and without signs of cognitive dysfunction at study inclusion. As easily accessible biomarkers for CCD are needed in veterinary medicine, we also investigated if systemic levels of Aβ-peptides, cytokines, or inflammatory markers were significantly higher in dogs with CCD compared to dogs with no or mild cognitive impairments (MCI).

Materials and Methods

Study Design and Procedure

The study was designed as a prospective longitudinal cohort study and was carried out at the Department of Veterinary Clinical and Animal Sciences, University of Copenhagen from February 2012 to April 2015. Client-owned dogs were recruited consecutively through the community practice and neurology referral clinic at the University Hospital for Companion animals.

The animals were treated according to the EU directive on handling and protection of animals used for scientific purposes (2010/63/EU) and the study was approved by the Ethical Committee of the Department of Veterinary Clinical and Animal Science, University of Copenhagen, Denmark. Informed and written consent was obtained from all owners.

The inclusion criteria was that dogs should be more than 8 years old and exclusion criteria were brain diseases other than CCD or concurrent medical problems that could possibly mimic signs of cognitive impairment. The inclusion period was open from February 2012 to March 2013 and the study period ended in May 2015.

The study design implicated a baseline assessment at inclusion (E_0), and 3 planned subsequent evaluations which served to monitor cognitive status over time: Six months after inclusion (E_1), 12 months after inclusion (E_2), and 24 months after inclusion (E_3). All investigations were performed by the principal investigator (TS) to secure a structured and consistent evaluation of all dogs.

The baseline assessment E_0 served to recruit dogs and furthermore to categorize the study population into 3 groups; a cognitively normal non-CCD group, a group with MCI, and a cognitively impaired CCD group. All dogs had a medical and cognitive evaluation including a clinical and neurological examination and assessment of body condition score. The auditory and visual system was evaluated as is standard in a full neurological examination. Hearing was tested by evaluating if the animal responded to sudden and unexpected sounds. Vision was evaluated by observing how the dog navigated in an unfamiliar environment and by testing the menace response which includes evaluation of all visual pathways. Additionally, the owner was instructed to report any changes of behavior in the home environment which could indicate a hearing or vision problem. The clinical evaluation also included collection of blood samples for complete blood count, serum biochemistry (inclusive of C-reactive protein and fibrinogen), thyroid profile and quantification of plasma Aβ-peptides and circulating cytokines. Further diagnostic work-up such as urinalysis, abdominal sonography, echocardiography, computed tomography (CT), magnetic resonance imaging (MRI), or cerebrospinal fluid analysis (CSF) were performed as indicated at the discretion of the principal investigator. Cognitive evaluation was based on face-to face interviews with the owners using the Canine Cognitive Rating Scale (CCDR)[9] (Table S1) and a supplementary CCD screening questionnaire[10] (Table S2). To address signs of fear and anxiety, 2 additional standardized questions were given to the owners. An interview would last for a minimum of 30 minutes. Answers were recorded for each dog in a separate file. The owners were encouraged to record their dog's behavior on video and the video observations were included in the assessment of cognitive status when available. Dogs were distributed into the non-CCD group, the MCI group and the CCD group based on the scoring results from the CCDR. According to the CCDR, a total score below 39 classifies a dog as having a normal cognitive status (non-CCD), a score of 40–49 classifies a dog as being at risk of developing CCD (MCI), and a total score above 50 classifies a dog as having CCD. The owners were asked to consent to donate the dog for postmortem examination if the dog was euthanized by the principal investigator during the study period.

Evaluations at 6 months and 24 months (E_1 and E_3) were conducted as structured telephone interviews using the CCDR questionnaire[9] and a supplementary CCD screening questionnaire.[10]

Evaluation at 12 months (E_2) was performed as a clinical control visit and included the same investigations as performed at E_0 except for quantification of thyroid hormones, plasma Aβ-peptides and circulating cytokines. For dogs that were euthanized or died spontaneously during the study period, the last investigator-client contact was at time of death or at the first coming prescheduled contact.

Blood Sampling and Analysis

Blood samples were collected from the cephalic or jugular vein. The hematologic and biochemical profiles (including C-reactive protein, fibrinogen, and thyroid hormones) were analyzed at the Central Laboratory, Department of Veterinary Clinical and Animal Sciences, University of Copenhagen.

Blood samples for Aβ and cytokine quantification were collected into vials containing EDTA, centrifuged ($2{,}500 \times g$, 15 minutes, 4°C), and plasma was immediately separated, snapfrozen on dry ice and stored at −80°C until batch analysis. $A\beta_{40}$ and $A\beta_{42}$ was measured using commercially available ELISA sandwich kits; Human βAmyloid(1–40) II and Human/Rat βAmyloid(42) High-Sensitive.[a] The applied capture antibodies for the N-terminal portion of human $A\beta_{40}$ and $A\beta_{42}$ are monoclonal anti-$A\beta_{1–16}$ (BAN50) and monoclonal $A\beta_{11–28}$ (BNT77), the C-terminal detection antibodies are HRP-conjugated anti-$A\beta_{1–40}$ (BA27) and anti-$A\beta_{35–43}$ (BC05), respectively. All samples were initially diluted 1:1 in urea (8 M) in order to monomerize the Aβ fibrils and reveal more epitopes to the detection antibody. Addition of urea to the samples did not have any influence on the performance of the ELISA kits from *Wako*.

N-terminal pyroglutamate-modified Aβ was quantified using the specific ELISA kit; Amyloid-β N3pE-42.[b] Capture antibody for this assay is antihuman Aβ(38–42) and the detection antibody is HRP-conjugated anti-Aβ N3pE (8E1). All samples were run in duplicates and optical density (OD) values were measured at 450 nm using an ELISA plate reader.[c]

Plasma concentration of IL-2, IL-6, IL-8, and TNF_α were simultaneously measured with a commercially available canine-specific multiplex immunoassay which employs an electrochemiluminescence detection technology.[d] The provided protocol for custom assay was used with no major modifications. The lowest detectable limit specified in the data sheet for IL-2, IL-6, IL-8, and TNF_α were 7.6, 2.4, 1.3, and 0.17 pg/mL, respectively.

Statistical Analysis

Descriptive analyses of the results obtained from the questionnaires and the additional questions regarding anxiety were carried out stratified by cognitive status. One-way analysis of variance (ANOVA) with post-tests (Tukey's multiple comparison test) was used for comparisons of parametric data from the 3 groups and Kruskal-Wallis test with post-tests (Dunn's multiple comparison test) was used for ordinal variables. Because of low sample sizes Fischer's exact test was applied for analysis of contingency tables containing 2 or 3 categorical variables. Correlations were assessed graphically as well as by Pearson's or Spearman Rank correlation coefficient where appropriate.

Survival curves, median survival time, and 95% confidence intervals were obtained by the Kaplan-Meier method and differences in survival were tested by the log-rank test. Survival time was counted from the day of birth to the day of death or right censored at end of the study. Outcome registration ended first of May 2015, outcome was defined as euthanasia primarily because of behavioral changes as a consequence of CCD or euthanasia/death because of other reasons.

Statistical significance was defined as $P < .05$. All statistical analyses were conducted using commercial statistical software.[e]

Results

Descriptive Data

A total of 57 privately owned dogs, 32 females and 25 males of various breeds, and ranging from 8 to 15 years (108–197 months) were enrolled in the study during the inclusion period. A total of 6 dogs were excluded, 3 dogs because of systemic disease, and 3 dogs because of inadequate owner interviews and thereby insufficient data, leaving a study population of 51 dogs. All dogs had a normal clinical and neurologic examination. No deficits of vision or hearing that could account for the cognitive changes were detected. Based on the scoring results from the CCDR, 21 dogs were categorized in the non-CCD group, 17 dogs in the MCI group, and 13 dogs in the CCD group at E_0.

The non-CCD group represented 14 different breeds and 4 mixed breeds with an age range of 106–197 months. The MCI group represented 10 different breeds and 4 mixed breeds with an age range of 108–192 months and the CCD group represented 7 different breeds and 4 mixed breeds with an age range of 127–192 months.

There were no significant differences with respect to sex distribution, weight, and body condition score between the cognitive groups at inclusion (Table 1). A significantly higher age was found for the dogs in the CCD group compared to dogs in the non-CCD and MCI groups ($P = .04$).

Fifteen dogs (10 dogs with CCD, 2 dogs with MCI and 3 dogs with no signs of CCD) had a postmortem examination of the brain using the trimming protocol for evaluation of large-sized brains.[18] Aside from cortical thinning and ventricular enlargement which were present in a number of CCD brains, no macroscopic lesions were detected.

Table 1. Association between sex, weight, body condition score, age, and cognitive status.

	Non-CCD (n = 21)	MCI (n = 17)	CCD (n = 13)	*P*-value
Sex				
Female	12	11	5	.25[a]
Male	9	6	8	
Weight (kg)				
Mean ± SD	18.9 ± 9.2	19.6 ± 9.0	13.9 ± 8.7	.20[b]
Body condition score (1–9)				
Median	6	4	7	.11[c]
Lower quantile	5	4	4.5	
Upper quantile	7	6	8	
Age (months)				
Mean ± SD	148 ± 22	149 ± 22	167 ± 19	.039[b]

CCD, canine cognitive dysfunction; MCI, mild cognitive impairment. Categorization in cognitive groups is based on the total canine cognitive ratings scale score.

P-values is calculated by: [a]Fischer's exact test, [b]ANOVA, [c]Kruskal-Wallis test.

Clinical Phenotype of CCD

Table 2 shows the distribution of impaired behavioral items for the 3 groups. The most frequent signs displayed by dogs in the CCD group were "pacing/wandering with no direction or aim" and "staring blankly at the walls or floor" as 91.7% of dogs displayed this behavior once a week or more frequently. "Avoiding being patted" and "difficulty finding dropped food" was observed in 75% of dogs in the CCD dogs. Supplementary questions revealed that separation anxiety or irrational fear to well-known objects/situations was observed in 33 and 58.3% of dogs in the CCD group, respectively.

The below signs were significantly more prevalent for dogs in the CCD group than for dogs in the MCI group: "avoids contact or being patted by the owner" ($P < .001$), "much less active compared to 6 months ago" ($P < .05$), "difficulty finding dropped food" ($P < .05$), and "walks into doors or walls" ($P < .05$).

The proportion of dogs with affected behavioral items in the CCD group was significantly higher than for the proportion of dogs with affected items in the non-CCD group for all behavioral items except for "separation anxiety" ($P = .16$), "irrational fear to well-known object or situations" ($P = .052$), and "fails to recognize family members" ($P = .36$).

Changes in Cognitive Status over Time

When analyzing changes in the CCDR score over time for the individual dogs, we found that in the non-CCD group 7 dogs (33%) progressed to the MCI group from baseline assessment to death or end of study. Two of these dogs progressed further from MCI to CCD. For the MCI group, 4 dogs (22%) progressed into a status of CCD from baseline assessment to death or end of study. No dogs evaluated as CCD during the study changed to a non-CCD or MCI status during the study. Four dogs with CCD at E_0 and the 2 dogs which progressed from non-CCD to CCD displayed an

Table 2. Frequency distribution of number of dogs (and percentages) with affected behavioral items in the non-CCD, MCI, and CCD group.

Category	Items	Non-CCD (n = 21) n (%)	MCI (n = 16) n (%)	CCD (n = 12) n (%)	*P*-value[$]
Spatial orientation Disorientation Awareness	Gets stuck behind objects and is unable to get around (happens once a week or more frequently)	1 (4.8)	7 (43.8)	6 (50)	1.0
	Walks into doors or walls (happens once a week or more frequently)	1 (4.8)	1 (6.3)	6 (50)	*
	Stares blankly at walls or floor (happens once a week or more frequently)	4 (19)	9 (56.3)	11 (91.7)	.09
	Disoriented at home	0	2 (12.5)	4 (33)	.43
Memory	Fails to recognize familiar people or pets (happens once a week or more frequently)	0	0	1 (8.3)	.43
	Indoor urination/ defecation in areas previously kept clean happens much more compared to 6 months ago	0	1 (6.3)	5 (41.7)	.057
Activity—apathy	Avoids contact or being patted by the owner (happens once a week or more frequently)	3 (14.3)	1 (6.3)	9 (75)	***
	Much less active compared to 6 months ago	1 (4.8)	1 (6.3)	6 (50)	*
Impaired olfaction	Difficulty finding dropped food in more than 31% of times	0	4 (25)	9 (75)	*
Locomotion	Paces up and down or wanders with no direction/purpose (happens more than once a week)	2 (9.5)	13 (81.3)	11 (91.7)	.61
Anxiety	Separation anxiety arisen after 8 years of age	2 (9.5)	0	4 (33.3)	***
	Irrational fear to well-known objects/situations	5 (23.8)	2 (12.5)	7 (58.3)	*
Learning and memory	Decreased ability/slow to learn new tasks	4 (19)	6 (37.5)	9 (75)	**
	Decreased ability to perform known tasks	0	6 (37.5)	7 (58.3)	.45
Sleep-wake cycle	Sleeps at day and restless at night	0	5 (33)	5 (41.7)	.70

CCD, canine cognitive dysfunction; MCI, mild cognitive impairment. Categorization in cognitive groups is based on the total canine cognitive ratings scale score.

Data were derived from the inclusion visit (C_0) as we wished to include answers from the first time the owner were given the questionnaires. Note that for 2 dogs (1 dog in the MCI group and 1 dog in the CCD group), specific data from the canine cognitive dysfunction rating scale are missing at inclusion, therefore these dogs are excluded in this analysis.

[$] *P*-values corresponding to statistical significant differences between the MCI and the CCD group.

*$P < .05$, **$P < .01$, ***$P < .001$.

increasing CCDR score with time. In 8 dogs (61.5%) in the CCD group had died or were euthanized within four months after the date of inclusion therefore no follow-up evaluation was possible. The CCDR score and time of death for the individual dogs from inclusion to end of study period is illustrated in Fig 1.

Survival Analysis

At the last contact (C_3), 41 dogs (82.4%) were dead and 8 dogs (15.7%) were still alive. Two dogs (3.9%) were lost for follow-up between E_0 and E_1. For the survival analysis, the dogs were divided into 2 groups,

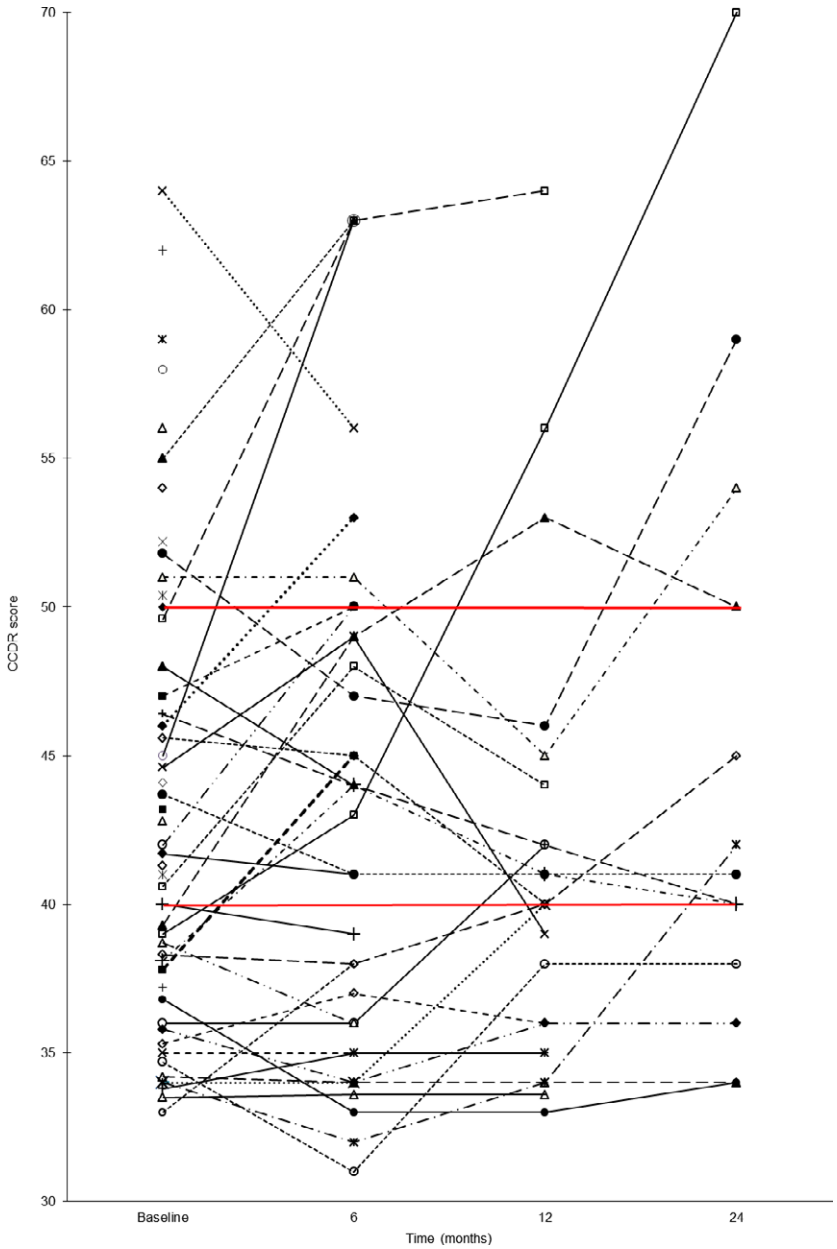

Fig 1. The canine cognitive dysfunction rating scale score and time of death for the individual dogs from inclusion to end of study period. The red lines indicate the cut-off values between cognitive groups. Note that for dogs which died between 2 preplanned assessments, time of death will appear in the figure at the next preplanned evaluation. At 24-month evaluation, 5 dogs had died between the 12 months and the 24 months evaluation and 8 dogs were still alive, thus evaluation was accomplished for 13 dogs in total.

a control group which consisted of 33 dogs that had non-CCD or MCI at baseline evaluation and no progression of cognitive status from non-CCD/MCI to CCD from baseline to death/end of study. The survival of this group was compared to the CCD group which consisted of 19 dogs that was diagnosed with CCD at baseline evaluation or had progressed to develop CCD during the study. Median survival time was 4,844 days for the control group and 5,367 days for CCD dogs (log-rank test *P*-value of .62). The mean follow-up time from inclusion to death for this study was 406 days (range from 0 to 1,113 days).

Plasma Biomarkers

There were no significant differences in concentrations of biochemical and hematological parameters between the non-CCD, MCI, and CCD groups (data not shown). The mean Aβ concentrations and $A\beta_{42}/A\beta_{40}$ ratio for each group are shown in Table 3. Concentration of plasma $A\beta pN_3$-42 was nonquantifiable as all samples were below lower limit of detection (7.75 pg/mL). Levels of $A\beta_{40}$ correlated well with $A\beta_{42}$ level across the study groups ($r = 0.59$, $P < .0001$).

Plasma concentration of both $A\beta_{40}$ and $A\beta_{42}$ varied considerably between dogs in all 3 groups, as denoted

by the high standard deviations (SD). The CCD group revealed significantly higher levels of plasma $A\beta_{42}$ levels ($P < .05$) than the MCI and the non-CCD group (Fig 2).

Nevertheless, individual values of $A\beta_{42}$ measurements showed considerable overlap between the 3 groups. Within the CCD group, a wide dispersion of the $A\beta_{42}$ measurements was present resulting in an apparent distribution into 2 clusters. One cluster of 7 CCD dogs had $A\beta_{42}$ levels above 95 pg/mL and for the other cluster consisting of 6 dogs, the $A\beta_{42}$ levels were less than 80 pg/mL. No significant difference in CCDR score was evident between the 2 clusters of CCD dogs. An increased plasma level of $A\beta_{40}$ ($r = 0.39$, $P < .005$) and $A\beta_{42}$ ($r = 0.31$, $P < .05$) were positively correlated with the CCDR score. $A\beta_{40}$ levels or the $A\beta_{42}/A\beta_{40}$ ratio was not significantly different between groups.

Concentrations of CRP, fibrinogen, and IL-8 were quantified in all dogs. IL-2 and IL-6 could not be quantified in plasma from 56 and 33% of the dogs, respectively, as measurements were below the lower limit of detection. Measurements of plasma TNF_α could not be quantified or were just above lower detection limit in all dogs. Dogs with non-quantifiable concentrations of IL-2 and IL-6, and all TNF_α measurements were excluded from the statistical analysis. There were no significant differences in concentrations of any of the systemic cytokines, C-reactive protein, or fibrinogen between dogs with CCD and dogs with no or MCI (data not shown).

Discussion

This study investigated cognitive function in a cohort of senescent dogs in a prospective longitudinal study design. The clinical phenotype associated with cognitive dysfunction in older dogs has been compared to humans suffering from Alzheimer's disease (AD),[4–8,19,20] and our study supported that striking similarities exists when evaluating the clinical appearance of affected dogs. The study documented that some dogs will develop signs of cognitive dysfunction with increasing age, whereas others remain cognitively healthy. Clinical signs of cognitive decline in dogs with CCD are cumulative and will worsen over time. This is also the case for humans developing (AD).[21,22] Based on the scoring results from the CCDR, the categories which were most affected in dogs with CCD were social interaction, activity, spatial orientation, and sleep-wake cycle, consistent with other studies.[2,11,13] With respect to specific clinical signs, "aimless wandering," "staring blankly into space," "avoiding being patted," and "difficulty with finding dropped food" were the most common signs displayed by dogs with CCD (Table 2). Decreased recognition of familiar people was an uncommon sign in CCD dogs in the present study. This is in accordance with that has been reported in 2 previous studies,[2,16] but in contrast to another study which reported that decreased recognition of familiar people was present in 60% of dogs with severe behavioral changes and thus a dominant sign of CCD.[9] The discrepancy between studies is most possibly explained by variations in the age and disease severity displayed by the animals included in the study populations. Furthermore, a factor which hampers comparisons between studies is that different questionnaires which do not necessarily address the same behavioral categories have been used to assess cognitive status.

Except for the CDS checklist,[1] the CCDR is the only CCD screening questionnaire which has also incorpo-

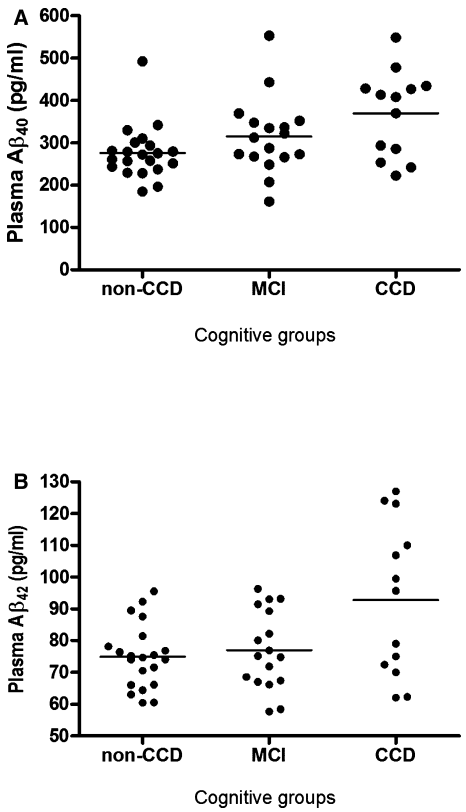

Fig 2. Plasma $A\beta_{40}$ and $A\beta_{42}$ measurements for individual dogs stratified by cognitive status. MCI, mild cognitive impairment; CCD, canine cognitive dysfunction.

Table 3. Concentration of plasma $A\beta_{40}$ and $A\beta_{42}$ (pg/mL) and $A\beta_{42}/_{40}$ ratios.

Group	$A\beta_{40}$ (pg/mL)	$A\beta_{42}$ (pg/mL)	$A\beta_{42}/A\beta_{40}$
Non-CCD (n = 21)	276.1 ± 63.0	74.9 ± 10.0	0.28
MCI (n = 17)	314.8 ± 89.4	77.0 ± 12.3	0.26
CCD (n = 13)	369.3 ± 100.9$^{*\text{non-CCD}}$	92.8 ± 24.0$^{**\text{non-CCD, MCI}}$	0.26

CCD, canine cognitive dysfunction; MCI, mild cognitive impairment. Categorization in cognitive groups is based on the total canine cognitive ratings scale score.

*$P < .01$ to the non-CCD group; **$P < .01$ to the non-CCD group, and $P < .05$ to the MCI group.

rated "difficulty finding dropped food" as an indicator of decreased olfaction. This sign was prevalent in CCD dogs in this study population. Decreased olfaction has previously been associated with CCD[9] and our results support that this sign could be a possible clinical indicator for CCD. Olfactory dysfunction and more specific impaired odor identification have also been reported for patients with AD and is presently investigated as an early marker of preclinical AD.[23–25]

Only few studies exploring CCD have investigated anxiety and fear as signs of significant interest.[1,2] This is quite surprising given that behavioral changes related to fear, phobias and anxiety have previously been reported to be prevalent signs by owners of senior dogs.[26] Such signs are presumably comparable to agitation and anxiety which is well known in humans with MCI and AD.[27,28] Our research group has previously shown that anxiety and fear are common in dogs with CCD,[2] which was why we also found it of importance to investigate such signs in this study. We found that separation anxiety and irrational fear to well-known objects/situations were present in 33% and 58% of dogs with CCD, respectively. This is even higher than the one previously reported[2], and emphasize both the importance of including questions addressing anxiety in future CCD screening questionnaires and the need to treat dogs with such troubling problems.

We used the CCDR for assessment of cognitive status as it is very useful for monitoring the progression of cognitive dysfunction.[9] A progression of the CCDR score over time was documented to occur in dogs from all groups (non-CCD, MCI, and CCD) and some dogs progressed from having mild cognitive disturbances (MCI) to displaying convincing signs of CCD. Our results supports that a preclinical stage of CCD is present in a subset of aged dogs. As previously reported, some dogs with MCI will develop CCD, whereas others will never progress.[2] In humans, such a symptomatic pre-dementia phase is recognized as MCI due to AD.[29] Only a proportion of human patients identified with mild cognitive deficits experience successive worsening of cognitive impairment, which eventually develops into fulminant AD.[29,30] For 2 dogs with a normal cognitive status at baseline assessment, a significant progression of the CCDR score was reported at E_1 and the CCDR score was further increased at E_2, now categorizing the dogs as CCD. Both dogs were evaluated at E_2, where no other medical causes than CCD could explain the rapid progression of cognitive impairment. This study demonstrates how the rate of progression of cognitive impairment can be variable between individuals and compares to the progressive phases of dementia severity reported for human AD.[29,31]

In this study, median survival time for dogs with CCD was not significantly different from dogs which experienced healthy aging. We have previously investigated survival with CCD[2] where CCD dogs had a longer survival time than non-CCD dogs. We speculated that this was possibly because of a close investigator-client contact which motivated the owners to keep dogs with CCD despite their cognitive deterioration. Because of the longitudinal design of this study, the investigator-client contact was even more consistent and in theory this might influence survival time for dogs with CCD even more positively. However, it might simply be that dogs with CCD do not experience a reduced life span because of the fact that the disease debut is late in life and because the human-animal bond is strong.

It is of great importance to search for diagnostic tests which can support a clinical suspicion of CCD and detect cases of MCI. We investigated selected biomarkers and found that plasma $A\beta_{42}$ was significantly higher in the groups of CCD dogs compared to the MCI and non-CCD groups. There was more variation in the level of $A\beta_{42}$ in the group of CCD dogs compared to the MCI and non-CCD group. This might imply that some CCD dogs have an increased level because of some underlying causes or that there is simply more variation in the CCD group. However, we do not have full explanation for this variation and further investigations including a larger study population is needed in order to examine this finding in more detail. The present study was a small-scale study and studies with more statistic power including serial plasma and possibly CSF measures would be beneficial to investigate if $A\beta_{42}$ is truly a relevant blood biomarker for CCD. Our results are supported by a previous study which reported significantly higher plasma $A\beta_{42}$ levels in cognitive impaired companion dogs compared to age-matched controls.[16] However, they also reported that dogs with severe cognitive impairments had lower $A\beta_{42}$ level than dogs with MCI, which was not the case in this study. There are several restrictions when comparing the results from the 2 studies. One striking difference is the substantially higher levels of both $A\beta_{40}$ and $A\beta_{42}$ and the lower variation in measurements recorded in our study. This difference might be explained by Aβ peptides binding to carrier proteins in plasma thus masking the epitopes and underestimating true Aβ values.[32] To minimize the risk of epitope masking, we pretreated the plasma samples with urea. Another factor adding to discrepancy between ELISA measurements from different studies could be because of diversity of the applied antibodies, exhibiting varying sensitivities and sometimes poorly defined specificities.[33]

A meta-analytic review concluded that it is not possible to establish a clear relationship between plasma Aβ peptide levels and clinical measures of AD severity.[33] Thus, a model of differential longitudinal changes in plasma $A\beta_{42}$ levels in cognitively stable human subjects versus those who go on to develop AD dementia has been proposed.[33] Individuals that will develop AD have a higher baseline $A\beta_{42}$ than cognitively stable individuals and the plasma levels will increase gradually during the MCI phase. At the conversion point from MCI to AD plasma $A\beta_{42}$ levels will diminish. Aβ levels in cognitively stable individuals will increase slightly with age.[33]

We speculate that the finding of higher plasma $A\beta_{42}$ levels in the CCD dogs compared to the cognitively unimpaired dogs in this study reflects that dogs with CCD are not as cognitively impaired as people with AD

and thus may correspond to early AD or the MCI phase. To gain a better understanding of plasma Aβ across the cognitive spectrum, longitudinal studies designed to include multiple time points for Aβ quantifications and where the enrolled dogs have the same age at inclusion would be beneficial. Validation against other biomarkers and histopathology would also be interesting.

The role of neuro-inflammation in disease progression of AD has been extensively studied. Fibrils of Aβ are believed to induce microglia activation with subsequent release of both pro- and anti-inflammatory mediators.[34–36] Complement factors, acute phase proteins, reactive oxygen species and cytokines such as IL-6 and TNF_{α} are considered among the most prominent neurotoxic factors.[35] Circulating cytokine concentrations in dogs with CCD have not been examined previously. Several of the cytokines investigated in this study could not be quantified although internal controls (spike recovery) were within acceptable limits and calibrations were valid. Although our results do not show a significant difference in circulating cytokine concentrations in dogs with CCD, a role for cytokines in the pathogenesis of CCD cannot be excluded. Local changes in the cerebral cortex of cytokine expression might possibly be more pronounced than reflected in the systemic concentrations.

Conclusions

This study documented that CCD is a progressive condition where the course of disease varies between individuals and thus compares to the progression through successive phases in human AD. Dogs with CCD commonly display specific clinical signs such as aimless wandering, staring blankly into space, avoiding being patted, and difficulty with finding dropped food. Furthermore, signs related to anxiety and unexplained fear is also common and should be addressed in future CCD screening questionnaires. Plasma $A\beta_{42}$ was found to be highest in dogs from the CCD group and thus may represent an interesting plasma biomarker which, however, needs further investigation.

Footnotes

[a] Wako Pure Chemical Industries, Ltd., Japan. Cat. no. 298-64601 and 292-64501

[b] Immuno-Biological Laboratories Co., Ltd., Gunma, Japan. Cat. no. JP27716

[c] Multiscan FC, Fisher Scientific UK Ltd., Leicestershire, England

[d] Canine Proinflammatory Panel 3 Assay, Meso Scale Discovery®, Rockville, MD

[e] GraphPad Prism 4.03 for Windows, GraphPad Software, La Jolla, CA

Acknowledgment

Conflict of Interest Declaration: Authors disclose no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in Supporting Information:

Table S1. Questions and scores included in the Canine Cognitive Dysfunction Rating Scale developed by Salvin and co-workers.[9]

Table S2. Questions and scoring method included in the questionnaire developed by Rofina and co-workers.[10]