Preliminary study on CCD in canine patients: Neurofibrillary tangles and ApoE4 in combination with an antioxidant fortified diet

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Abstract

Canines are known for their natural development of age-related neuropathological brain lesions, such as beta amyloid deposits as senile plaques, cerebral amyloid angiopathy and lipofuscin. Presence of these features in combination with cognitive decline and behavioral alterations is considered canine cognitive dysfunction (CCD). Studies have provided evidence of the beneficial effect of an antioxidant fortified diet in CCD patients and have connected these effects to reduced brain pathology. CCD in dogs shares similarities with Alzheimer's disease (AD) in humans, such as beta amyloid and tau pathology. The canine has therefore been suggested as a possibly promising animal model of AD. In scientific research devoted to AD, ApoE has suggested to be an important apolipoprotein in the disease pathology. Aims of the present investigation were to expand the knowledge of CCD and to support the view of the dog as a valuable animal model of AD. First, by studying the occurrence of neurofibrillary tangles (NFT's). NFT's and dense content containing plaques have not been consistently demonstrated in the dog. Second, by exploring the ApoE4 immunoreactivity in dogs. From a veterinary standpoint, this study focuses on the possible correlation between the quantity of ApoE4 staining and antioxidant dietary treatment. Therefore 11 of the 12 canines available for immunohistochemistry were divided into 3 groups. One group containing old demented dogs, the second group housed the old demented dogs with dietary treatment and the third group consisted of the control dogs. Histopathological evaluation of NFT's with the Bodian silver staining method resulted in the finding of tangles in four of the six demented dogs. Immunoreactivity of canine ApoE4 with human antibodies was found in all of the nine elderly dogs. A trend for lower ApoE4 quantities was demonstrated, with evident differences between the ApoE4 amount in the groups old demented and non-demented old control dogs. The distinction based on the ApoE4 staining in macrophages (microglia) proved significant.

Introduction

Alzheimer's disease (AD), the most common form of dementia in humans, is a chronic neurodegenerative disease which causes progressive impairment of memory and other cognitive functions (Hoozemans et al. 2009). The term dementia refers to a cognitive decline severe enough to compromise a person's daily function. The first clinical sign of dementia is a loss of memory, which goes beyond the amount that can be expected to be part of the normal aging process. However, as the disease slowly progresses, an increasing number of other intellectual functions will start to be affected, such as attention, language, visuospatial skills and problem solving (Kelley, Petersen 2007). The clinical picture of most patients with AD agrees with this pattern of symptoms, by showing a prominent memory disturbance followed by the involvement of other cognitive domains and by developing behavioral alterations (Teri, Larson & Reifler 1988, Drevets, Rubin 1989, Burns, Jacoby & Levy 1990, Rosen, Zubenko 1991, Fleming, Adams & Petersen 1995).

Histopathologically, Alzheimer's disease is characterized by two neuropathological hallmarks: extracellular amyloid deposits, also known as amyloid or senile plaques and intracellular neurofibrillary tangles (NFT's). NFT's are composed of hyperphosphorylated tau protein (Selkoe 2001a, Morris 1997). Tau is a microtubule-associated and stabilizing protein primarily expressed in the central nervous system (Hoozemans et al. 2009, Spires-Jones et al. 2011). During disease pathogenesis, normal tau becomes hyperphosphorylated, undergoes a conformational change to paired helical and straight filaments of abnormal tau and these filamentous or fibrillar forms of tau aggregate into mature NFT's (Kuret et al. 2005). Amyloid plaques consist mainly of aggregated amyloid beta (Aβ) peptides, that are proteolytic fragments of amyloid β precursor protein (APP), derived from physiological cleavage and are believed to accumulate in the AD brain due to failure of efficient Aβ clearance (Selkoe 2001b, Tanzi, Moir & Wagner 2004, Zlokovic 2008). It has been furthermore suggested by D'Andrea and Nagele (D'Andrea, Nagele 2010), that there are several different types of amyloid plaques in AD

brains. The most common types are classified in literature as dense core and diffuse, based on their characteristic appearance in immunohistochemical preparations of postmortem AD brains. The increasing cerebral Aβ concentration, which will eventually lead to the formation of plaques, is thought to create synaptic dysfunction and neuronal cell loss. Recent studies however, report the soluble oligomers as the most toxic Aβ species to synapse and neuron (Matsunaga, Suenaga 2012, Gao et al. 2010, Haass, Selkoe 2007), where the classical amyloid hypothesis holds the aggregation of amyloid beta into insoluble beta-sheet fibrils responsible (Hardy, Selkoe 2002). The most pressing argument in favor of the current view is the fact that cognitive decline in AD patients is not correlated with the levels of senile plaque formation or insoluble Aβ formation. Instead it correlates with the levels of synapse loss and the levels of soluble amyloid beta (Tomiyama 2010). A slightly corresponding development can be seen in the way neurofibrillary tangles are believed to be part of Alzheimer's disease pathology. It is well known that tau dysfunction can lead to neurodegeneration, since there are several neurodegenerative diseases that share a tau filaments containing pathology, such as Pick's disease and frontotemporal dementia with Parkinsonism (Spires-Jones et al. 2011, Goedert, Spillantini 2006). But whether NFT's or soluble hyperphosphorylated tau levels should be held responsible for the neurotoxicity is currently being debated. According to Spires-Jones et al. (Spires-Jones et al. 2011), recent studies challenge the classical view of tangles as toxic species in the brain. Although NFT formation correlates to some degree with neuronal loss and cognitive decline in AD, soluble forms of tau have been found to correlate with neuronal and synaptic dysfunction in several transgenic mouse models (Berger et al. 2007, Hoover et al. 2010, Santacruz et al. 2005, Sydow et al. 2011). Additional neuropathological findings in AD patients are cerebral amyloid angiopathy (CAA), which constitutes Aβ depositions in arteries, arterioles and capillaries, and lipofuscin (Revesz et al. 2003). Lipofuscin is a lipopigment and derives from material resulting from cell damage or disease and/or from the normal processes of renewal of cellular constituents. As both features are known to

Alzheimer's disease, they are also possible findings in non-demented elderly subjects (Mountjoy et al. 2005, Eurelings et al. 2010).

AD can appear either sporadically or can be transmitted in an autosomal dominant fashion. In the rare familial forms of AD, clinical mutations in the APP gene ultimately lead to the development of both Aβ plaques and NFT (Oddo et al. 2003, Gotz et al. 2004). This genetic evidence mainly provided the strongest support for the amyloid cascade hypothesis, which predicts that the accumulation of Aβ is the trigger for all cases of AD (Hardy, Selkoe 2002). As mentioned earlier, the most supported reason for this accumulation is an unsuccessful clearance of amyloid beta. A promising view on how this Aβ metabolism may become disabled, is provided by several pieces of recent evidence. Amongst others, Hirsch-Reinshagen et al. (Hirsch-Reinshagen, Burgess & Wellington 2009) suggested that abnormal cholesterol metabolism may constitute a key factor in the pathogenesis. Lipids within the central nervous system (CNS) are transported on high-density lipoprotein (HDL) particles. ApoE is the predominant apolipoprotein in the CNS and acts to scaffold the formation of HDL particles, which promote the proteolytic degradation of soluble forms of Aβ in mouse models (Donkin et al. 2010, Jiang et al. 2008, Cramer et al. 2012). ApoE facilitates and significantly enhances the proteolytic degradation, both within microglia (the resident macrophages of the central nervous system) and in the extracellular milieu, through the action of two distinct classes of proteinases (Jiang et al. 2008). The lipidation status of ApoE has proven to be a corresponding factor, since well-lipidated forms of ApoE facilitate the Aβ degradation more efficiently (Wahrle et al. 2005, Koldamova, Staufenbiel & Lefterov 2005, Hirsch-Reinshagen et al. 2005). The effect of ApoE on microglial Aβ clearance is additionally isoform-dependent. Previously published genetic evidence showed the possession of ApoE4 alleles to entail an increased AD risk and an earlier start in a gene dose-dependent way, whereas inheriting the ApoE2 allele is protective (Corder et al. 1993, Corder et al. 1994). In accordance, the study by Jiang et al. (Jiang et al. 2008) showed that ApoE2 represented the strongest effect whereas ApoE4 was significantly less efficient in promoting

the degradation of soluble Aβ. Furthermore, the molecular mechanisms by which ApoE influences intracellular proteolysis of Aβ, is suggested to be through reducing cellular cholesterol levels (Lee et al. 2012).

In summary, intensive scientific research increasingly assembles knowledge about the not fully understood pathogenesis of Alzheimer's disease. The most common animal models used in these studies are mice, which have to be genetically altered to obtain AD corresponding features. Although the usefulness of transgenic mice is generally accepted, it has been hypothesized that additional relevant information on the physiopathology of AD could be acquired from other natural non-transgenic models (Sarasa, Pesini 2009). Dogs might be a useful model. As in the case of human medical technologies, improvements in veterinary medicine and husbandry have contributed to a longer lifespan of our domestic animals, but with a greater incidence of age-related neurodegenerative diseases (Sarasa, Pesini 2009, Landsberg 2005). The age-related syndrome found in canines is referred to as canine cognitive dysfunction (CCD). To define this condition, the disease is characterized by progressive problems with learning, memory and spatial awareness, as well as changes in social interactions and sleeping patterns (Landsberg, Hunthausen & Ackerman 2003, Landsberg, Araujo 2005, Neilson et al. 2001). CCD occurs with an equivalent spontaneous development and variability as AD in humans and accordingly, several matching neuropathological features have been found (Papaioannou et al. 2001, Yu et al. 2011). As reported by Cumming et al. (Cummings et al. 1996) the level of beta amyloid accumulation correlates with cognitive dysfunction in the canine. Several studies provide evidence of the occurrence of cognitive decline and behavioral alterations as a result of a CCD (Colle et al. 2000, Head et al. 1998, Adams et al. 2000, Rofina et al. 2006).
Because of the way this age-related syndrome naturally reproduces key aspects of AD, it can be seen as the canine equivalent of Alzheimer's disease (Salvin et al. 2011). However, dense core neuritic plaques and neurofibrillary tangles have not been consistently demonstrated in the dog.

Various research did not show positive results on the occurrence of these specific plaques and tangles in canines (Cummings et al. 1996, Wegiel, Wisniewski & Soltysiak 1998, Wisniewski et al. 1996, Pugliese et al. 2006), where other research projects did (Papaioannou et al. 2001, Rofina et al. 2006). For a more conclusive illustration of these currently controversial features, further research is required. In light of recent developments suggesting ApoE to be a significant factor in the Alzheimer's disease pathology, reports on the presence and workings of ApoE in canines may as well help the expand our knowledge of CCD and AD. Referring to the way ApoE is thought to facilitate the Aβ degradation in human patients. ApoE has been marked in cerebral amyloid deposits and human NFT's by Namba et al. (Namba et al. 1991). Additionally, an immunohistochemical study of Uchida et al. (Uchida et al. 1997) showed canine senile plaques and CAA to be intense immunoreactive for ApoE. This data can therefore be motivating towards research aimed at the significance of ApoE in the CCD pathology.

In the present study, a silver staining and immunohistochemical method are used to acquire better insight into the development, presence and workings of neurofibrillary tangles and ApoE4 in the canine brain. In order to increase the understanding of Alzheimer's disease by exploring the value of the dog as an animal model, but especially, to gain insight into canine cognitive dysfunction, from the veterinary point of view. As a therapeutic intervention, an antioxidant fortified diet has been proven to be beneficial to CCD patients (Milgram et al. 2002, Christie, Opii & Head 2009, Head 2007, Cotman et al. 2002). A study by Pop et al. (Pop et al. 2010) showed a significant reduced senile plaque load in the parietal and entorhinal cortex and significantly decreased levels of soluble Aβ in the prefrontal cortex. As an extension of these theories and the previously named research by Uchida et al. (Uchida et al. 1997), this study will also look for potential effect of antioxidant fortified food on the molecular pathways of ApoE4. In order to increase the welfare of pet dogs by aspiring to improved veterinary care.

Materials and methods

Subjects and clinical data

In this study, canine brain specimens of nine old demented and four non-demented control dogs of various breeds (Table 1) were examined. These dogs became available to this research via several private veterinary practices, located all over the Netherlands. Results were obtained from all subjects (Table 1) individually and in addition, in order to measure the effect of an antioxidant fortified diet, three groups of dogs were formed out of these subjects (Table 2). As established in multiple studies, the breed does not influence the outcome of pathological lesions in the brain of a canine (Colle et al. 2000, Borras, Ferrer & Pumarola 1999, Rofina et al. 2004). The dogs were euthanatized for diverse reasons and donated by their owners for research. Of all dogs, the medical report was studied and necropsy was performed in order to discover any physical cause that could possibly interfere with CCD pathology (Rofina et al. 2006). All canines were clear of such physical issues, but one that was admitted to the current study suffering from renal disease. This concerns dog number six. During life, the dementia score of all dogs but subject number one were calculated by applying a questionnaire adapted by Rofina et al. (Rofina et al. 2006) to diagnose Alzheimer-like changes in canine behavior. Alzheimer-like changes are considered to be established at a score over 10. Brain tissue from the two young control dogs was additionally obtained. Both canines previously served as a negative control dogs in another research project and were three months old. Necropsy was routinely preformed and the brain tissue was fixed in buffered 4% formaldehyde. A sample of human brain tissue of a demented patient, to act as a positive control section when evaluating immunohistochemistry, was donated by the VU University Medical Center, Amsterdam. Diagnosis of Alzheimer's disease was earlier defined in this patient.

Table 1. Breed, gender and age of the dogs employed in this study

Nr.	Breed	Gender	Age (years)
1.	Collie	F	23
2.	Dachshund	M*	15
3.	Mixed-breed	M*	18
4.	Mixed-breed	F*	15
5.	Beagle	M	14
6.	Mixed-breed	M*	14
7.	Mixed-breed	M	13
8.	Golden Retriever	F*	16
9.	Mixed-breed	M	11
10.	Samoyed	M*	13
11.	Mixed-breed	M	16
12.	Great Dane	M	0
13.	Great Dane	M	0
F = female, M = male, * = neutered			

Histopathology

Brain tissue of the first six dogs was neuropathological evaluated. The assessment was performed on formalin-fixed, paraffin-embedded tissue from the frontal and parietal lobes. The presence of dense, dense core and diffuse plaques, NFT's, lipofuscin and CAA was determined by light microscopy, using Bodian silver staining (Uchihara 2007). The Bodian staining was routinely executed and carried out at the Academic Medical Center, University of Amsterdam.

Quantification

Bodian silver staining was studied in the brain tissue samples, white and grey matter and meninges, to detect plaques, NFT's, lipofuscin and CAA. The positivity of each item was graded by a semiquantitative scoring system: counting the amount of stained blood vessel, diffuse plaques, tangles and lipofuscin for each section. The researcher scoring these alterations was kept blind about the data of the brain samples. The results were classified as

negative (= 0), few (= 1), moderate (= 2) and severe (= 3) (Rofina et al. 2004, Rofina et al. 2003). Negative referrers to zero visualization of an item, one to three observations quantifies as few, three to 10 as moderate and more than 10 alterations in a section equals a severe score. Furthermore, the presence of dense and dense core plaques was noted.

Immunohistochemistry

Dogs involved in this part of the research, were studied individually and divided into groups (Table 2).

Sections of formalin-fixed paraffin-embedded tissue (5 µm thick) were mounted on Superfrost plus tissue slides. To inhibit endogenous peroxidase activity, slides were immersed in 0,3% H_2O_2 in methanol for 30 minutes at room temperature (RT). Antigen retrieval was performed in 85% formic acid for 15 minutes RT and slides were subsequently rinsed in tape water and PBS. Sections were treated with normal horse-serum (1:10 dilution) in PBS for 30 minutes in a humid chamber at RT. Incubated with the primary antibody (mouse anti-ApoE4 in 1:200 dilution) in PBS the slides stayed overnight at 4°C. After being washed with PBS/Tween-20, the slides were incubated with biotin-conjugated horse-anti-mouse IgG (1:125 dilution) in PBS for 45 minutes. Followed by rinsing with PBS/Tween-20, incubation with ABC/PO complex solution in PBS for 45 minutes and washing with PBS. Color was developed using 3,3'-diaminobenzidine substrate solution as chromogen. Sections were counterstained with hematoxylin and mounted using Eukitt. As a positive control, one sample of human brain tissue was used. Negative controls were obtained by omitting the primary antibody and by using brain tissue from both the young control dogs.

Quantification

In the investigation of ApoE4, several positive structures were counted and graded in brain tissue from the frontal and parietal lobes: larger blood vessels, capillaries, diffuse, dense and dense core plaques and macrophages (microglia), located in white and grey matter and meninges. In this

quantification, no differentiation was made between the different kind of plaques. Each positive item was graded and classified by a semiquantitative scoring system, entirely equal to the one used for the quantification of the histopathology. And again, the researcher scoring these alterations was kept blind about the data of the brain samples. An additional score was provided per slide, based on the overall staining of ApoE4, referred to as the total score.

Table 2. Groups of dogs (old demented, old demented with dietary treatment and non-demented) formed in this study

Nr.	Breed	Gender	Age (years)
	Old demented dogs		
3.	Mixed-breed	M*	18
4.	Mixed-breed	F*	15
7.	Mixed-breed	M	13
10.	Samoyed	M*	13
	Old demented dogs with dietary treatment		
5.	Beagle	M	14
6.	Mixed-breed	M*	14
8.	Golden Retriever	F*	16
	Non-demented control dogs		
	Old		
9.	Mixed-breed	M	11
11.	Mixed-breed	M	16
	Young		
12.	Great Dane	M	0
13.	Great Dane	M	0
F = female, M = male, * = neutered			

Statistical Analysis

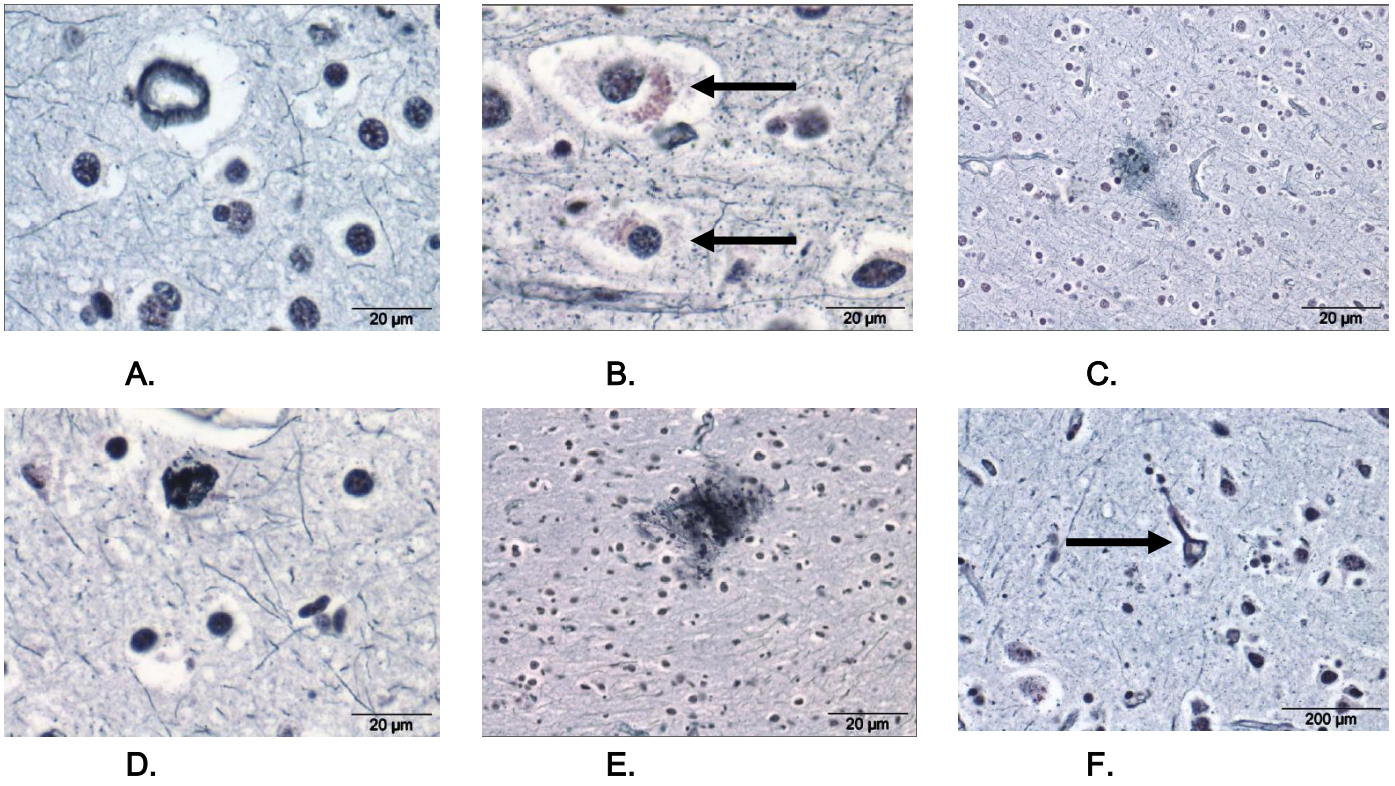
SPSS 16.0 for Windows was used for the statistical analysis of the data. One-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons was used to test for differences between groups. Correlation analysis was done using the Pearson parametric test. For establishing significance, a P value of <0,05 was taken.

Results

Histopathology

Brain tissue of the first six dogs available for histopathological staining, showed positive results for CAA, lipofuscin and diffuse plaques in white and grey matter and meninges (Figure 1A-C). There was no difference detected in these results between dogs or between the frontal and parietal lobes in the score of neuropathological features. In addition, dense and dense core plaques were distinguished in the frontal and parietal lobes of four canines (Figure 1D-E). This concerns the dogs numbered one and four to six. The same canines demonstrated staining of neurofibrillary tangles (Figure 1F), however this staining was not confined to the slides positive for dense and/or dense core plaques. Meaning that there were slides showing positivity for NFT's and no
dense and/or dense core plaques. The amount of NFT's or plaques with dense content was explicitly less than the amount of CAA, lipofuscin and diffuse plaques and did not appear to increase with age. The NFT's did show a distinctive location, since all of the tangles were located in parietal lobes. Statistically, no significant correlation was noted between the occurrence of NFT's and the dementia score. Dementia scores and silver stain intensity of the neuropathological lesions previously mentioned, are summarized in Table 3.

Figure 1. Histopathological brain lesions found in this study



Histological silver staining in cerebral brain tissue of demented canines: CAA in cortex (A), lipofuscin in neurons in the cortex (B), diffuse plaque in cortex (C), dense plaque in cortex (D), dense core plaque in white matter (E) and NFT in neuron in the cortex (F).

Table 3. Summary of histopathological features & dementia scores found in this study

Nr.	CAA	Lipofuscin	Diffuse plaques	Dense/dense core plaques	NFT's	Dem. Score
1.	3	3	3	1	1	Unknown
2.	3	3	3	0	0	19
3.	3	3	3	0	0	37
4.	3	3	3	1	1	37
5.	3	3	3	1	1	21
6.	3	3	3	1	2	17
7.						21
8.						18
9.						10
10.						15
11.						10
12.						10
13.						10

Dem. score = Dementia score

0 = negative, 1 = few, 2 = moderate, 3 = severe

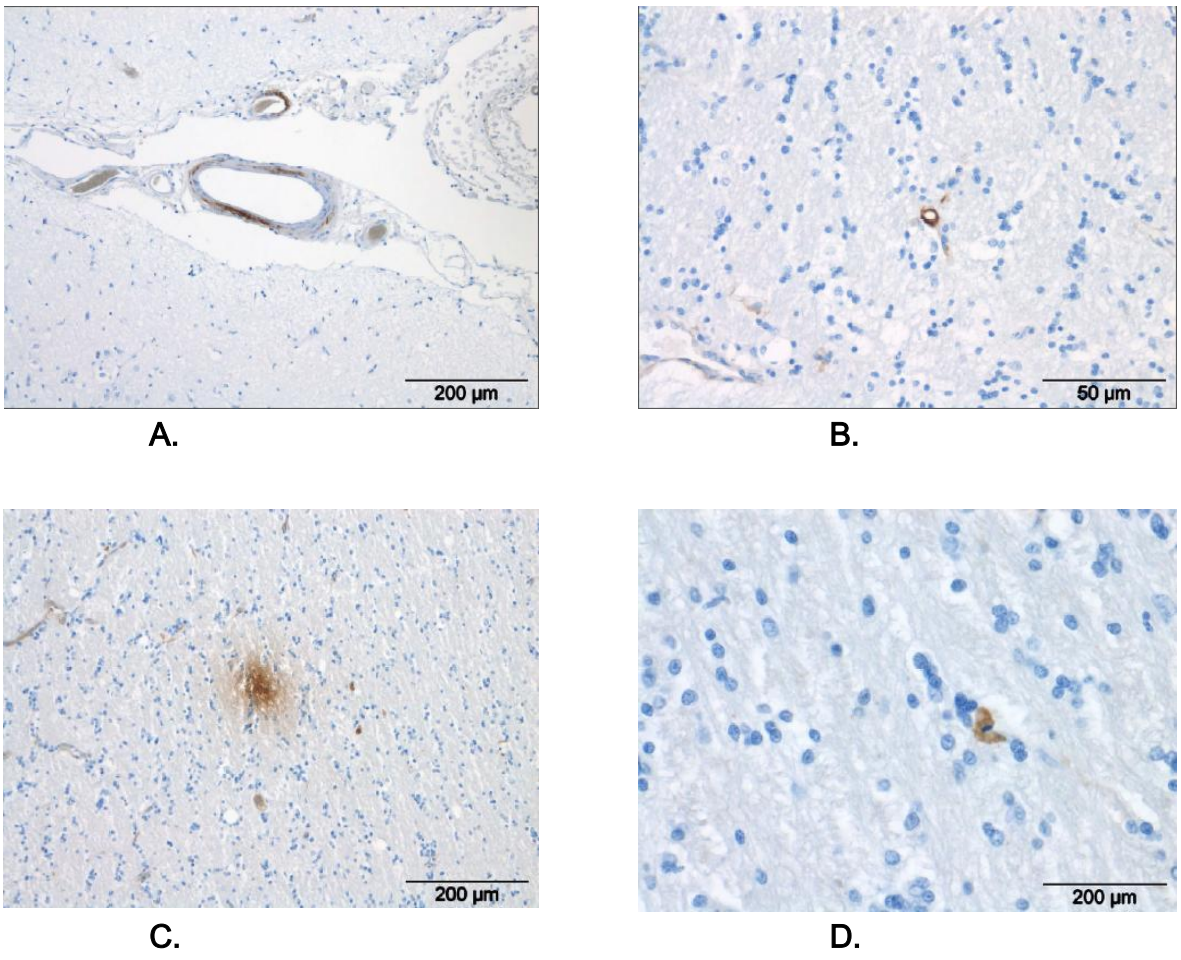
Immunohistochemistry

The ApoE4-immunostaining labeled larger blood vessel, capillaries, plaques and macrophages in 11 of the 13 examined canine brains (Figure 2A-D). The positive structures were distributed throughout several cortical layers, including the frontal, parietal and occipital area. Immunoreactivity of larger blood vessels, followed by the immunoreactivity of capillaries was more encountered than of positive plaques or macrophages. The human positive control showed an intense immunoreactivity of all structures and every one of the three negative controls, including the two young control dogs, demonstrated a negative staining result. The total staining classification of ApoE4 had no apparent relationship to the brain localization or to age. It furthermore did not significantly correlate with the dementia score or with the amount of NFT's.

By dividing dogs into three groups: old demented, old demented with dietary treatment and non-demented, special attention could be paid to the effect of

an antioxidant fortified diet on the quantity of ApoE4 present in the canine brain. As stated earlier, 11 dogs showed ApoE4-immunoreactivity. Table 4 illustrates the classification of the total immunoreactivity and of the examined structures individually. Comparing the groups, the ApoE4 staining results revealed that there is a difference in the amount of ApoE4 between old demented, old demented with dietary treatment and non-demented old control dogs. The non-demented young control dogs were not included in the comparisons, to avoid age to be a contributing factor. Group data of the total ApoE4 staining classification is plotted in Figure 3. However, only the difference in ApoE4 positive plaques reached a statistically significant level ($P < 0.05$), comparing the old demented dogs with the non-demented old canines. In all differentiations performed, these two groups differed the most, followed by the statistic distinction between the group of regular old demented dogs and the patients with dietary treatment.

Figure 2. Histopathological brain lesions found by immunoreactivity in this study

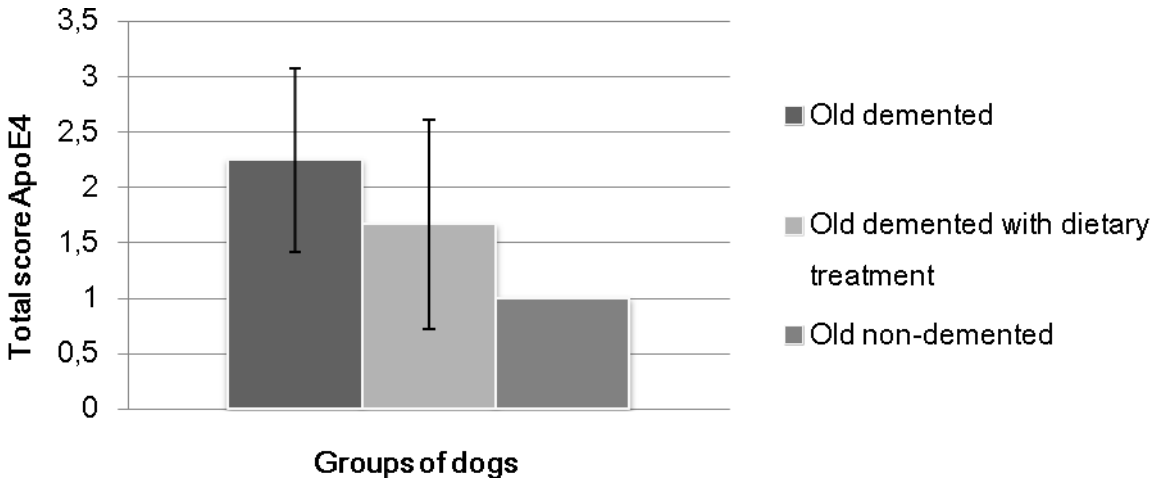


Histopathological immunostaining in cerebral brain tissue of demented canines: ApoE4 in larger blood vessels in meninges (A), ApoE4 in capillary in white matter (B), ApoE4 in diffuse plaque in white matter (C), ApoE4 in macrophage in white matter (D).

Table 4. Summary of immunoreactivity of histopathological features found in this study

Nr.	Larger blood vessels	Capillaries	Plaques	Macrophages	Tot. Score
	Old demented dogs				
3.	2	3	2	3	3
4.	2	2	1	2	2
7.	2	1	1	1	1
10.	3	3	1	3	3
	Old demented dogs with dietary treatment				
5.	1	0	0	1	1
6.	1	0	0	0	1
8.	3	3	1	3	3
	Non-demented control dogs				
	Old				
9.	1	1	0	0	1
11.	1	1	0	0	1
	Young				
12.	0	0	0	0	0
13.	0	0	0	0	0
Tot. score = Total score 0 = negative, 1 = few, 2 = moderate, 3 = severe					

Figure 3. The differences of the total amount of ApoE4 between groups of dogs found in this study



Discussion

The present study examined a few age-related findings of the canine brain regarding plaques with dense content, NFT's and immunoreactivity for ApoE4. To emphasize the CCD pathology in the six (for histopathology available) brain tissues, CAA, lipofuscin and diffuse plaques were also scored. As expected, every one of these neuropathological features was recurrently found in the silver stained slides (Table 3). This prospect was based on the earlier determined diagnosis of CCD in the six dogs by Rofina et al. (Rofina et al. 2006), on literature confirming the Bodian silver staining method to be suitable for showing these brain lesions (Uchihara 2007) and on research outcomes connecting the presence of CAA, lipofuscin and/or diffuse plaques to CCD and/or normal brain aging (Cummings et al. 1996, Colle et al. 2000, Rofina et al. 2006, Borras, Ferrer & Pumarola 1999). In this respect, canine patients with canine cognitive dysfunction and human patients suffering from AD are in agreement. Amyloid or senile plaques are a characteristic of the disease. CAA and lipofuscin are frequently found in AD patients as well as in non-demented elderly subjects (Selkoe 2001a, Morris 1997, Mountjoy et al. 2005, Eurelings et al. 2010).

Not quite as expected, or as supported in writings, are the findings of neurofibrillary tangles and dense content containing plaques (Wisniewski et al. 1996). There is research stating that tangles do not develop in canines (Cummings et al. 1996) or that only tau phosphorylation can be detected (Yu et al. 2011, Cummings et al. 1996, Wegiel, Wisniewski & Soltysiak 1998, Pugliese et al. 2006). However contradictory findings, where canine NFT's were demonstrated, have also been published (Papaioannou et al. 2001, Rofina et al. 2006). Papaioannou et al. (Papaioannou et al. 2001) showed neurofibrillary tangles by using the Gallyas silver staining method as well as by immunohistochemistry with three different tau antibodies. NFT's were also found in the current research, all located in the parietal lobe. This suggests neurofibrillary tangles to be more frequent in the parietal area of the canine brain. In a study by Rofina et al. (Rofina et al. 2006) most of the detected

lesions, though this study did not include NFT's, were also located in the parietal lobe. These inconsistent research results may indicate NFT's findings to be very dependent on the method use for visualization. Even the differentiations between diverse silver staining methods can be sufficient to create various staining outcomes, since the Gallyas (Papaioannou et al. 2001) and Bodian method resulted in detection, where the Bielschowsky method (Yu et al. 2011) did not. In opposition, the presence of NFT's may as well be determined by the susceptibility of individual dogs towards developing the pathology (Sarasa, Pesini 2009). In this research, no significant correlation was found between the quantity of tangles and the dementia score. In humans, NFT formation has been suggested to correlate to some extent with neuronal loss and cognitive decline, whereas soluble forms of tau have been found to correlate with neuronal and synaptic dysfunction in several transgenic mouse models (Berger et al. 2007, Hoover et al. 2010, Santacruz et al. 2005, Sydow et al. 2011).

In light of recent scientific progress surrounding ApoE and its proposed value in the AD pathology, the current study focused on and found immunoreactivity of canine ApoE4 with human antibodies. Consistent with expectation and a study done by Uchida et al. (Uchida et al. 1997), this result indicates a molecular similarity of ApoE4 between humans and dogs. It has been previously published that ApoE4 is present in human tangles (Namba et al. 1991). In this study no correlation was found between the amount of NFT's and the total ApoE4 staining score in dogs. Genetic evidence that a total of 80% of familial and 64% of sporadic AD late onset cases have at least one ApoE-ε4 compared to the 31% of control subjects, has been obtained via human medicine (Corder et al. 1993). Correlation of ApoE4 with the dementia score was not achieved by the current research, in contrast with the original theory. Species-based or individual-based differentiations could provide an explanation for these outcomes. It should also be mentioned that one of the dogs diagnosed with CCD was suffering from renal disease, which has been described to cause behavioral changes that can be mistaken for behavioral alterations as a result of CCD (Summers, Cummings & DeLahunta

1995). The negative ApoE4 staining results in the two young control dogs suggest the absence of age-related neurological features such as CAA and senile plaques. Especially since Uchida et al. (Uchida et al. 1997) showed canine senile plaques and CAA to be intense immunoreactive for ApoE. In accordance with this study, where ApoE4 immunoreactivity was found in all of the 11 elderly dogs. Research has suggested that beta amyloid, the main component of amyloid plaques (Selkoe 2001a, Hardy, Selkoe 2002) and CAA (Revesz et al. 2003), does not begin to accumulate until approximately eight years of age (Head 2011). As expected on the basis of this information, the three month old Great Danes were therefore successful as fully negative controls, where the two older canines showed some physiological immunoreactivity for ApoE4 in larger blood vessels and capillaries. Overall, immunoreactivity of larger blood vessels, followed by the immunoreactivity of capillaries was more encountered than of positive plaques or macrophages. This finding can be related to human literature stating CAA deposits to be intimately associated with smooth muscle cells (SMC's) or SMC-derived pericytes and Aβ to be internalized by SMC's via ApoE-containing lipoproteins (Urmoneit et al. 1997, Prior, Wihl & Urmoneit 2000). Combined with a study by Jiang et al. (Jiang et al. 2008) on mice where ApoE4 was shown significantly less efficient in promoting the Aβ clearance, these results propose a potential isoform-dependent ApoE-mediated pathway, responsible for Aβ clearance in the brain.

Studies have suggested that an antioxidant fortified diet in canines suffering from CCD, may lead to cognitive improvement (Milgram et al. 2002, Christie, Opii & Head 2009, Cotman et al. 2002). There is persuasive evidence in humans that the cognitive and behavioral deficits characteristic of AD arise, in part, from impaired synaptic function due to soluble forms of Aβ (Cramer et al. 2012). Pop et al. (Pop et al. 2010) were able to significantly connect antioxidant treatment with modest effects on levels of soluble and insoluble Aβ species and assembly states, plus a significantly decreased Aβ plaque load in several brain regions. During the current research a trend for lower ApoE4 quantities was demonstrated. The differences between the ApoE4

amount in the groups old demented and non-demented old control dogs were especially evident, with a significant distinction in macrophages. Interestingly, the group differences for all of the parts studied in this manner (larger blood vessels, capillaries, plaques, macrophages and total score), were repeatedly the lowest in the comparison between the old demented and the old demented with dietary treatment dogs. The conclusion that might be formatted from these data, is that the groups old demented with dietary treatment and old non-demented are closer to each other and that the antioxidant treatment might have participated in this result. Age could not be a contributing factor to this phenomenon, since the average of the group old demented with dietary treatment is situated closer to the average of the group old demented than the group with non-demented old control canines.

As a result of information obtained by this and previous scientific research projects, NFT's and dense content containing plaques should be seen as possible causes of CCD. Further research is needed to investigate more reliable methods of demonstrating these aggregations of abnormal tau protein, given that the methods currently in use lead to inconsistent outcomes. Additionally, expending this preliminary study and studying the neurotoxicity of NFT's and soluble hyperphosphorylated tau peptides in larger group canines could be promising, because of its associations with cognitive decline in human AD patients. It can provide more insight into de CCD pathology and may even lead to a better understanding of Alzheimer's disease in humans, since the dog is suggested to serve as a superior animal model of this disease (Sarasa, Pesini 2009, Cummings et al. 1996).

Immunohistochemistry results of the current project support this statement regarding the dog as an animal model for Alzheimer's disease. The molecular similarity of ApoE4 between humans and dogs reveals the canine as a more complete animal model of AD, with a wider biochemical similarity with humans then just concerning cerebral β-amyloidosis (Uchida et al. 1997). Furthermore, it suggests that ApoE4 may be involved in the proposed positive mechanism(s) of an antioxidant diet concerning the neuropathological

features of CCD (Milgram et al. 2002, Christie, Opii & Head 2009, Head 2007, Cotman et al. 2002, Pop et al. 2010). As can be said of the results of the histopathology, the outcomes of the immunohistochemistry in this preliminary study are indications that when repeating this study in a larger sample size of dogs, significant results can be found. Further research relating to the workings and effects of ApoE, including ApoE4, in canines suffering from CCD with or without dietary treatment could thus be considered useful.

To summarize, by acquiring a positive staining for NFT's and ApoE4 in CCD patients and a sign that ApoE4 might be connected to the beneficial effects of an antioxidant diet as proposed in scientific literature (Milgram et al. 2002, Christie, Opii & Head 2009, Head 2007, Cotman et al. 2002), this study may have brought the knowledge of CCD a little closer towards the actual truth. And in addition may have added value to the canine as an animal model for AD. This article therefore suggests the dog as an important animal model to use in the continuance of scientific research concerning Alzheimer's disease. Future research of canine cognitive disorder could be truly helpful to support healthy ageing in canines and humans.

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