

DOGS WITH COGNITIVE DYSFUNCTION SYNDROME: A NATURAL MODEL OF ALZHEIMER'S
DISEASE

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ABSTRACT

In the search for appropriate models for Alzheimer's disease (AD) involving animals other than rodents, several laboratories are working with animals that naturally develop cognitive dysfunction. Among the animals tested, dogs are quite unique in helping to elucidate the cascade of events that take place in brain amyloid-beta (A β) deposition aging, and cognitive deficit. Recent innovative research has validated human methods and tools for the analysis of canine neuropathology and has allowed the development of two different approaches to investigate dogs as natural models of AD. The first approach relates AD-like neuropathy with the decline in memory and learning ability in aged housed dogs in a highly controlled laboratory environment. The second approach involves research in family-owned animals with cognitive dysfunction syndrome. In this review, we compare the strengths and limitations of housed and family-owned canine models, and appraise their usefulness for deciphering the early mechanisms of AD and developing innovative therapies.

KEYWORDS: aging, Amyloid-beta, animal model, canine cognitive dysfunction syndrome, family dog, therapies.

Why a canine model of AD?

For years, the search for appropriate animal models for Alzheimer's disease (AD) has focused on a variety of manipulations to reproduce the disease in various species, such as yeast, *Drosophyla melanogaster*, *Caenorabditis elegans*, rodents, and rabbits, with a particular interest in obtaining knockout and transgenic animals (for a review, see [1]) research is currently performed in the mouse because its brain structure is somewhat similar to that of humans, and it is amenable to highly sophisticated genetic engineering. Because age-related cognitive decline is a common feature of most mammals, other laboratories have focused on the few species that naturally develop cognitive dysfunction with various Alzheimer-like characteristics. These animals include the monkey, polar bear, cat and dog [2-5]. Canine models are currently considered a useful intermediate between genetically modified mouse models and humans [6,7]. Although many of the transgenic AD models have provided insights into the molecular mechanisms of the pathology, none of them encompass all the cognitive deficits observed in AD (see Table 1). One of the first transgenic mouse AD models was the amyloid precursor protein transgenic mouse, PDAPP mouse, which develops age-dependent amyloid beta accumulation and deposition in both diffuse and fibrillar neuritic plaques in the hippocampus, cerebral cortex, and corpus callosum [8]. Other models with AD-like pathology are apolipoprotein E (ApoE) or Tau transgenic mice, and also mice in which other genes have been modified. In more sophisticated approaches, important information has been gained from crossing PDAPP and ApoE models and with mice lacking or over-expressing genes for beta-secretase, alpha-secretase, or the insulin-degrading enzyme [9,10]. All these models have provided valuable information on AD pathology and on the possibility of developing new treatments such as secretase inhibitor therapy or immunotherapy.

However, transgenic models are plagued with limitations, especially in terms of their ability to fully represent a correspondence between mutations and disease. For example, in mouse models, mutations in amyloid precursor protein (APP) and presenilin frequently trigger only A β plaques, whereas in humans these mutations lead to AD with plaques, tangles, and severe brain atrophy. These models also give information mainly on the pathogenic factors of inherited forms of the disease, whereas mammals' models characterized by cognitive deficit syndrome and AD markers are more likely to address the underlying pathology of the disease and identify therapeutic targets for innovative therapies.

As a large mammal, the dog has the advantages of a larger brain and larger cerebrospinal fluid (CSF) volume and, like the monkey, presents a diversity of cognitive assessments that are close to the human cognitive processes impaired in AD [11,12]. The correlation established between the progression of canine cognitive

dysfunction, A β plaque maturation, and several cellular and molecular AD markers confirms the dog as a natural model for the study of both AD and human brain aging [6] and should reduce the need of non-human primates. With regard to the numerous AD canine similarities, the APP, an iron-export ferroxidase [13], and the A β 1-40 and A β 1-42 peptides present a high homology in dogs and humans[2,14,15], and soluble A β peptide deposition in the form of senile plaques and cerebral amyloid angiopathy in the dog brain parallels that in humans. Canine presents the same characteristics of cortical neuronal loss but, in contrast to humans, its tau hyperphosphorylation is not accompanied by neurofibrillary pathology [16,17] probably due to differences between human and dog protein sequences. In any case, the deposition of A β is one of the earliest neuropathological occurrences in AD and the aging process, and since neurofibrillary tangles are common to a number of types of dementia, a central role of A β peptides is generally accepted and referred to as the “amyloid hypothesis” [18]. To test this hypothesis, which holds that soluble A β peptide accumulation in the brain extracellular space (forming soluble oligomers, fibrils and A β plaques, in equilibrium between blood and brain compartments) is responsible for neuronal dysfunction and degeneration, the only natural candidates at present are dogs over 7-8 years [16,19-22]. Because of that, dogs are quite unique to investigate the early events taking place in the diffuse A β plaque maturation and its relationship with cognitive deficit. Detailed explorations of the neurochemical cascade of toxicity, and the identification of the specific proteins involved in each step, are major lines of research [23,24] that can be profitably pursued in canines [25]. The same is true for the identification of the exact mechanisms that trigger sporadic AD. The correlation between chronic stress, the rate of AD incidence and the presence of A β found in clinical studies [26] can also be investigated in canine to decipher AD mechanisms and understand the physiological consequences of that correlation . Dogs also offer multiple druggable targets for the identification of novel disease-modifying agents interfering with A β production, oligomer formation, fibrils and plaque formation and maturation, the neurotoxic effects of these products or their clearance process [11,23,27-31].

Two different approaches are currently used to investigate the dog as a natural model of AD, each one applying a different cognitive assessment methodology. The first relates AD-like neuropathology with decline in memory and learning ability in aged dogs of a specified strain (in general, beagles) raised in highly controlled laboratory environments. The second investigates family animals (also named companion dogs, client-owned dogs or non-housed dogs) diagnosed with cognitive dysfunction syndrome (CDS), a well-defined clinical disease in aging dogs. Besides the diversity of breeds (including sex, size and genetic background) and family life conditions [32], CDS dogs represent a validated model for AD research [16]

(Figure1). This last approach has the advantage to rule out the need of a specific strain to be housed during years in controlled conditions.

In this paper, we first review the CDS, and then compare the features the canine models with special focus in the comparisons between behavioral modification of housed and family dogs. The robustness of these models to provide new insights into AD pathophysiology mechanisms is also discussed. Then we propose new targets for the development of new therapeutic approaches to early AD intervention.

The canine Cognitive Dysfunction Syndrome

With improved standards of veterinary care and the maintenance of the human-animal bond, the veterinary profession is treating a larger number of elderly animals than ever before, and elderly dogs are the most rapidly growing segment in many veterinary clinic populations. Current estimates suggest that there are more than 30 million senior and geriatric dogs over the age of seven years in the USA [33] than 15 million in Europe, accounting for approximately 30-40% of the total canine population in these geographical areas. Their owners tend to take a strong interest in their aging process.

As in humans, the canine aging process involves progressive, irreversible changes in the whole body, and is frequently associated with severe specific behavioral and cognitive deficits [34] that lead to changes in interactive, elimination or navigational behaviors that are not due to the primary failure of any organ system. When cognitive dysfunction is not due to a primary cause such as a brain tumor or infarct, its clinical diagnosis as CDS requires the presence of one or more of the following nine behavioral changes

- 1) Decreases or changes in reactivity to routine stimuli
- 2) Confusion or disorientation
- 3) Changes in elimination behaviors, ranging from sporadic inappropriate elimination to incontinence
- 4) Decreased interaction with owners
- 5) Increased irritability
- 6) Slowness in obeying orders
- 7) Alteration in sleep-wake cycles
- 8) Decreased responsiveness to sensory perception
- 9) Changes in the capacity to solve problems (e.g., increased frequency of getting stuck in corners or lost in the garden or house) and in general problems in performing previously learned

behaviors.

All these symptoms are included in CDS [35], which corresponds to the broad spectrum of behavioral problems equivalent to the definition of human dementia in the Diagnostic Criteria of Mental Disorders [36] stage 4-6 of the Global Deterioration Scale [37]. Some authors suggest a correspondence between aging dogs and human mild cognitive impairment [11]. In a study of 180 dogs with no identifiable health problems, 28% of owners of 11- to 12-year olds reported at least one category consistent with cognitive impairment and 10% in two or more categories; this figure rose to 68% for dogs of 15 to 16 years of age with owner reports of signs in at least one category and 36% in two or more categories [38]. Other studies suggest that the diagnostic rate is underestimated by veterinary neurologist [39].

In-vivo studies to assess canine cognitive deficit

Magnetic resonance imaging techniques and aging

To complete the cognitive assessment of aging dogs, magnetic resonance imaging (MRI) techniques are of major interest. They are currently applied to dogs in veterinary clinical practice to confirm a suspected lesion or to identify its extent or location, and also to diagnose central nervous system (CNS) diseases such as inflammation or brain anomalies. However, they are not commonly used for the prediction, diagnosis or follow-up of canine aging with cognitive dysfunction. The relationship between progressive canine brain atrophy and aging has been evidenced in several post-mortem studies [40-42] and recently confirmed in housed dogs, frequently beagles, using a variety of MRI techniques [43,44]. To determine whether progressive global cerebral atrophy is a marker of aging in dogs as well as in humans, an MRI study in companion dogs was performed with an adapted version of a simple human visual rating assessment (Fig.2). This new scale represents a reliable, rapid, universal method of canine brain atrophy measurement with the advantage that it does not require specialist supervision [45]. The results are similar to those of human brain aging and show that both hippocampal and progressive global atrophy correlate with aging. This reinforces the possibility of using housed and non-housed dogs to investigate the human cerebral atrophy process in greater depth.

CSF parameters and canine cognitive deficit

The correlation of CSF parameters with cognitive deficit reflects some of the central pathogenic processes of AD [46,47], and makes it possible to detect early dysfunctions of energy metabolism, proteosomal activity,

and lipid oxidation related with A β deposition. As such, the A β peptide binds to complex-1 NADH dehydrogenase [48] and increases mitochondrial dysfunction, oxygen free radicals and derived reactive oxygen species production [49]. These products result in the massive generation of damaged proteins with cross-linked polypeptides that not only resist proteolytic attack but also inhibit proteosomal activity, thereby establishing a deleterious cycle that may eventually result in the cell's demise [50]. Deficiency in the fatty acid beta-oxidation pathway is also associated with neurodegeneration, possibly due to binding of A β peptide to hydroxyacyl-CoA dehydrogenase [51] resulting in increased mitochondrial dysfunction (Fig.3). Finally, neuronal energy metabolism is fuelled by glucose and lactate specifically supplied by astrocytes due to the close neuronal-astrocyte cross-talk, whose CSF levels reflect the intensity of neurodegeneration and may serve as a marker of CNS activity [52]. For example, CDS dogs, the large glucose variability and the higher values of pyruvate and lactate and K⁺, only found in the group with severe cognitive deficit (SCD), relate to an impaired cerebral oxidative glucose metabolism that participates in the advanced cognitive impairment [32]. In a pathological situation, lactate constitutes a critical neuronal energy substrate with positive vasodilatory effects, and with no reason to be considered a noxious agent [53]. In fact, a new paradigm considers lactate to be a central neuroprotective agent [52,54]. In the group with light cognitive deficit (LCD) these values are normal, probably due to the capacity of adaptive mechanisms to compensate for a lower brain insult [55].

Post-mortem studies to assess canine cognitive deficit

A β diffuse plaque maturation, A β oligomers, neuronal vulnerability and glial reactivity

Senile plaques and the diffuse deposits of A β are considered the hallmarks of the aging dog [35,56]. Their presence in association with cognitive decline has led to their being proposed as the major factor implicated in neurodegeneration. Beginning around the age of eight, and increasing with age, the formation and maturation of diffuse deposits of A β can be observed by immunostaining throughout all canine cortical gray matter layers in a characteristic four-stage distribution that correlates with the severity of cognitive deficit in the dog.

This characterization of canine A β plaque composition, maturation and distribution, of tissue reactivity in terms of specific death or resistance of subsets of neurons, and of glial reactivity has produced similar results in housed beagles and non-housed dogs. Their brain section examination shows typical age-changes such as nerve cell loss, lipofuscin accumulation in neurons and the presence of corpora amylacea [57-60]. Like AD

patients, dogs with SCD present large, isolated, scarce calcium deposits – mostly hydroxyapatites – some of which localize within the astrocyte cytoplasm [61]. As shown in rat brain lesions [62], these deposits result from an excitotoxic process associated with the activation of microglia and astrogliosis, which participate in neuronal death.

Brain A β Distribution

Except for some limited reports of Congo red staining plaques, investigators generally agree that canine plaques are formed by A β 1-40 and 1-42, and are of the diffuse human type [63-66], rather than the beta-pleated-sheet-conformation (and so negative for Congo red and thioflavine stainings). As in humans, plaque distribution within the brain is heterogeneous and its abundance increases in parallel to the increase in cognitive decline. In all cases, canine prefrontal cortex is the main site of the plaque onset, followed progressively by the parietal, entorhinal and occipital cortices [67]. In these regions, A β plaques appear to act as the insult causing brain tissue neuronal and glial responses, leading to the dog's progressive cognitive decline [37,68].

In prefrontal cortex, A β precipitates are initially detected in low numbers in the deeper layers (V, VI), forming the dispersed plaques of stage I distribution; some can fuse together and sometimes extend to layers IV and III, taking on the cloud-like appearance characteristic of stage II distribution. The stage III pattern presents dense positive plaques localized mainly in the superficial layers (III, II), together with cloud-like depositions similar to those seen in stage II in the lower layers. In stage IV, the positive plaques observed in stage III extend throughout all cortical layers, but are smaller and more dense (Fig.4a). However, aside from these marked differences, all the plaques observed are of the *diffuse* human type and lack any cerebral neuritic component. Stage II-IV distribution also presents significant differences in the density and size of the A β plaques. For example, the mean plaque size falls from 5543.50 mm² (stage II) to 4618.48 mm² (stage III) and 1240.01 mm² (stage IV). These differences are also observed when data are adjusted for age [68].

In AD, the specific functional and pathological alterations are less severe in the cerebellum than in other brain areas, particularly the entorhinal cortex and hippocampus. Since dense core A β plaque formation has been associated with an acetylcholinesterase heterogeneous nucleator action [69], the relation between cerebellar pathology, acetylcholinesterase density and cognitive dysfunction has also been studied in family dogs [70]. In these animals, the late cerebellum involvement is evidenced by the absence of A β plaque. However, the highest acetylcholinesterase reduction correlates with aging and loss of granule cells, but the

cognitive deficit only with the loss of Purkinje cells. This result does not support an interaction between cerebellar acetylcholinesterase activity and A β deposition, and indicates that symptoms and clinical signs of canine cognitive dysfunction are mainly of non-cerebellar origin.

In AD patients, the levels of fibrillar oligomers correlate with Mini Mental State Examination (MMSE) scores and with A β plaque stages. These levels are high in frontal cortex regions, hippocampus, entorhinal cortex, transentorhinal cortex and cerebellum, all regions involved in AD. In aged dogs, no correlation has been found canine cognitive dysfunction and pre-fibrillar oligomers, but the possible association with fibrillar oligomers is yet to be studied [22].

Amyloid reactivity

Abundant reactive microglia is found within the AD neuritic plaques diffuse canine plaques are closely associated with microglia and astrocyte reactivities [49,71]. Together with cortical reactive astrocytosis, activated hypertrophic astrocytes over-expressing S100 β are generally found just outside the human AD plaque boundary with processes deep into the A β deposition. Because of this, S100 β has been considered an important pathogenic factor in the genesis and evolution of AD plaques. The hypothesis that the absence of canine dense-core A β plaques is due to differences in the neurotrophic effects of astrocytes has been explored in companion dogs. Classifications of dogs according to their cognitive deficit correlated with the relative abundance and stage of cortical A β plaques, and with the interaction with S100 β negative astrocytosis [68]. For example, stage IV plaques are closer to astrocytes, and astrogliosis correlates with diffuse plaque maturation, but in absence of any S100 β over-expression (Fig.5). Therefore, A β plaques might attract reactive astrocytes to participate directly in the tissue response, without a direct S100 β involvement.

With regard to the microglia reaction, only fine processes of ramified microglia are stained in dogs with cognitive deficit. Capillaries present intense staining that increases with aging and stage of A β deposition. Perivascular macrophages present a granular pattern of cytoplasmic staining. Few positively stained activated microglia cells are associated with dense core A β plaques and no significant correlation of any positively stained microglia cells with A β plaques has been established. So, the involvement of S100 β and microglia in the progression of AD does not seem to initiate in the very early stage of plaque formation. However, the lack of a significant microglial reaction associated with diffuse plaque maturation also described by other laboratories [72] should be considered with caution because monoclonal antibodies used for immunohistochemistry were not shown to be specific for dog microglia cell surface markers, nor cross

reacted with them. Research into this area is currently underway based on in vitro autoradiography analysis. At the cellular level, the specific vulnerability to neuronal death of the α -aminobutyric acid (GABA) cortical subset of interneurons, characterized by their calcium-binding protein content and relationship with A β deposition, has been investigated in companion dogs and compared with results from human AD and housed beagles [73]. In companion dogs younger than 8 years, the general distribution and cell typology of Parvalbumin (PV)-, calretinin (CR)-, and calbindin (CB)-positive interneurons is similar to those described previously for beagles [74], suggesting the presence of a common pattern that is not breed- or sex-specific. In old dogs with cognitive deficit the study demonstrates specific vulnerability among CB-immunopositive GABAergic cortical interneurons and resistance among PV-positive and CR-positive ones. As AD patients present similar data [75,76], a similar highly conserved role of these calcium-binding proteins characterized by their different capacity to buffer calcium may confer different levels of protection on their neuronal subpopulation in humans and dogs[77]. In addition, when analyzed for age-related loss and A β toxicity, aging appears critical for the fate of CB-positive neurons, with the amyloid deposition in stage II being a second key factor that renders these neurons vulnerable to death. So, the A β deposition in stage II, when dogs are 10 - 14 years old, may be the toxic factor that renders the subset of cortical CB-immunopositive neurons (GABAergic neurons) vulnerable to death. If so, this would explain why in AD these same neurons are also more vulnerable to death.

However, it remains to be established whether this amyloid deposition directly causes a Ca²⁺ dysregulation through glutamate receptor induced excitotoxicity, or whether it is the parallel increased level of A β oligomers that is responsible for this effect [22,78]. In AD, the correlation between plaque stage and levels of fibrillar oligomers also makes it difficult to clarify the precise cascade of events that controls specific neuronal fate [79].

In any case, this deleterious effect, not detectable in stage III or IV plaque deposition, may act as an early event in the pathological cascade leading to neuronal death and glial reaction. In the initiation phase, stage II (or the soluble toxic oligomers) may reinforce the ongoing aging process, increasing the generation of free radicals [80] and leading to more severe neuronal dysfunction. As found in AD, oxidative stress may also be related to high Zn²⁺ levels that inhibit APP ferroxidase activity [13]. In any case, these results confirm the view that aging in certain brain areas begins relatively early in adult life, and also corroborate the sequence of events described in the housed aged canine brain and proposed as the initiation phase of human brain aging [25], associated with a peak of oxidative stress and a dramatic drop in bcl-2 expression. In these

animals, Bax expression is also increased after 15 years of age, when stage II has terminated. The presence of oxidative stress markers like 4-hydroxynonenal in high concentrations detected by immunohistochemical techniques in the A β deposits, vascular wall areas, perivascular spaces and inside some neurons in aged canine brains confirms their participation in the same degenerative process [58].

Localization, distribution, and the relationship between A β deposition and tau hyperphosphorylation in companion dogs (Fig.4b) are not associated with increased active mitogen activated protein kinase (MAPK/ERKP), p38 kinase (p38-P) expression, or tau hyperphosphorylation in neighboring cell processes. Hyperphosphorylation, as revealed by the presence of phospho-specific antibodies, increases with age in individual neurons but does not correlate with cognitive impairment [16]. This negative result, also found by other laboratories [49], suggests that tau hyperphosphorylation is not directly involved in canine brain damage.

The question of why neurofibrillary pathology is absent in the aged canine brain remains controversial. As neuritic plaque formation is a multiple-step process that develops over decades, a simple explanation is that the dog life-span (usually not longer than 18 years) is too short to allow this formation. Another explanation for the lack of tangles relates to the specific pattern of excessive tau protein phosphorylation, since the canine tau peptide sequence differs from that of humans, and no paired helical filaments are formed [81]. Another explanation focuses on the accelerated time course formation of aggregates of cathepsin D and advanced glycation end products in canines, the scarcity of these structures in humans being explained by the fact that their biological aging process is slower [82]. In this situation, dogs may not develop tangles, and aging CB-immunopositive neuronal loss and A β deposition, together with these other anomalous structures, would drive cognitive decline. If this is the case, interventions to preserve CB-positive neurons and to avoid ROS action in the early stage of plaque formation, will be of major interest for reducing GABAergic cortical neuronal loss associated with aging. In any case, the redox state of the intracellular environment is particularly important, given its involvement in neurodegeneration and aging.

These numerous similarities found between canine and human brain argue for the incidence of common risk factors in their cognitive decline. The CB-positive neuronal loss in AD patients may reflect, at least in part, the early diffuse plaque boosting of the aging vulnerability effect. This early loss of GABAergic neurons would then modify a series of events and potentiate the excitotoxic process leading to increased neuronal loss, astrogliosis, microgliosis, reactive oxygen species formation and chronic brain damage with reactive amyloid plaque.

Behavioral studies in aged dogs

A fine-grained, graded cognitive assessment is necessary to ensure proper classification of each animal and adequate follow-up of its cognitive process. Numerous studies have applied different procedures to evaluate learning and memory deficits in aging dogs. All these procedures are based on two types of evaluation: the first, applied to housed dogs, assesses learning and evaluates tasks in the laboratory, and the second, applied to companion animals, is based on clinical explorations and surveys or interviews with owners whose responses are then scored.

These behavioral studies performed with one methodology or the other represent the true difference between the two paradigms of dog AD models. So, as other *in vivo* and post-mortem studies carried out in each type of canine model are similar, they will be reviewed as a whole, and the specific characteristics of each model commented on when appropriate.

Behavioral assessment of housed dogs

In the laboratory, several tasks have been selected due to their sensitivity for reflecting specific cortical circuits and/or specific brain regions functions. By means of a cognitive test apparatus (a canine adaptation of the Wisconsin General Test Apparatus) all laboratory behavioral tests (such as the object recognition memory task, delayed-non-matching-to-position task, delayed-non-matching-to-sample test), are conducted using food as a reward to motivate learning in the animal [83-86]. Aging dogs (mostly beagles) are diagnosed with substantial cognitive decline when they present reduced capacity in the learning of tasks such as object recognition memory [83], visuospatial learning and memory [87,88], allocentric spatial function [86], discrimination learning and discrimination reversal learning [89]. From all these studies two main conclusions have emerged: a) the detection of canine cognitive dysfunction depends on the cognitive process engaged, on the task used and on the relative level of difficulty; and b) the variability in the cognitive abilities of dogs increases with age. An important aspect that can limit the use of this canine model is the length of time required to perform each test, and the cost of their handling and housing: for example, a simple associative learning task may require up to two weeks to be completed, and more complex tests such as a memory test up to four months [83].

Behavioral assessment of companion dogs

In companion dogs, a clinical approach and surveys or interviews with owners, whose responses are then scored, can determine the presence of the characteristic signs of CDS. The animal's cognitive performance is appraised in only 15-20 minutes, but the appraisal depends on the ability of the veterinary neurologist to explain the various questions, and on the reliability of the owner's answers. The well-known disadvantages of interview usage must be taken into consideration in the final diagnosis, especially the reluctance of some owners to answer certain questions concerning their pet such as aggressiveness, the importance of the interviewer's experience, and the subjectivity of some answers.

In recent years, several questionnaires have been developed with specific items to classify dogs' behavior and to establish correlations with several neuropathological markers of aging or of cognitive dysfunction [19,32,39,90,91]. For example: *Colle et al.*[90] designed an easy-to-apply clinical scale (Evaluation of Age-Related Cognitive and Affective Disorders-ARCAD), based on owner interviews, which provides a global evaluation comparable to the clinical scales used in human practice such as the MMSE or the Activities of Daily Living. Canine evaluation with the ARCAD scale allows a good correlation between behavioral deficits related with maintenance behavior (eating, drinking, auto-stimulatory behavior, elimination behavior, sleep) and A β deposition. However, it does not correlate with environment-dependent symptoms (such as learned specific behavior, self-control, learned social behavior and adaptive capabilities), probably due to the difficulty of evaluating them through owner interviews, or because the loss of maintenance behavior has a better clinical value.

The Criteria for Evaluation of Dementia in dogs proposed by *Kiatipattanasakul et al.* [19] include several items for analysing the correlation between behavioral changes and apoptosis of neuronal and glial cells. These items do not include several important behavioral and cognitive aspects such as walking, posture, or sensorial symptoms (hearing loss, hypersensitivity to smell). To investigate the relationship between cognitive performance and markers of brain pathology (cortex atrophy, A β , oxidative damage, demyelination and accumulation of macrophages), *Rofina et al.* recently used a questionnaire to score the behavioral changes of aging dogs [72], and established high correlations with most of these pathological data. Their analysis suggested a key role for cellular and nuclear oxidative damage in behavioral changes.

Around the same time, in our laboratory we developed a new 16-item questionnaire to identify the discriminative events in early and late stages of cognitive dysfunction of aging dogs based on our expertise and on the human MMSE and The Diagnostic and Statistical Manual of Mental Disorders [32]. Detection of

dogs with LCD is important to help identify significant cellular and molecular events requiring investigation in early stages of AD. The questionnaire assesses the global and progressive decline of memory, cognition, and personality in canines of different age, sex and breed [32].

It was completed during the veterinary neurologist's interview of the owners, and the results were analyzed and compared with the data obtained directly from the clinical exploration. Because owners' understanding and the capacity of evaluation of the behaviors covered by these seven items were different, the similarity of the scores obtained in all dogs must be due to other factors. For instance, several items like eating, barking, drinking and self-control are easy for owners to understand but difficult to evaluate, and the item *aggressive* is difficult to qualify because of the owners' reluctance to acknowledge this behavior in their pet. The items "auto-stimulatory" and "learned social behavior" are difficult for owners to understand or evaluate. So, these seven items had to be removed, not because they reflect behaviors that do not change with aging and cognitive deficit, but because the difficulties in defining and evaluating them may distort their scores and mask their relevance.

The nine items finally selected cover a diversity of behaviors considered sufficient to evaluate the cognitive status of each dog and to ensure good discrimination between animals (Table 2). Items such as maintenance behavior (including elimination behavior and life rhythm) and others such as walking, posture, playful and exploratory behaviors, are easy to understand and evaluate. In contrast, except for interaction with other animals or with owners, the items identified as environment-dependent behavior, such as learning of specific behaviors and adaptive capabilities, are more difficult to understand and require explanations from the veterinarian. The validated nine-item test gives a similar classification of the dogs as the full 16-item version, is less time-consuming and has gained in accuracy and selectivity, especially for the classification of dogs with LCD. The scores suggest the existence of three groups of dogs that differ in age and cognitive status. Specific aspects like breed or sex did not influence the classification. So the test appears to be a valid tool for rapid, easy diagnosis of the initiation and progression of canine cognitive deficit. Dogs varying widely in terms of breeds and family environment are easily classified based on their age and cognitive status into three well-defined groups, namely young control (YC), LCD and SCD animals. Further characterization and comparison of each group allows the identification and follow-up of the early molecular and cellular aspects of the disease, and validation of new treatments. For example, this classification into three groups correlates with the relative abundance and stage of cortical A β plaques, even when data are adjusted for age.

A significant correlation between behavioral test scores and A β accumulation is also found in housed dogs,

with a strong correlation between errors in discrimination memory, reversal memory and spatial learning and A β load in frontal and entorhinal cortices [37,89]. In these dogs the stage of plaques correlates with the age [92]. **Table 3** shows these correlations in both canine models.

Overall, these data demonstrate that a fine-grained assessment of graded canine cognitive dysfunction is necessary to establish accurate correlations between the different central events that take place progressively in cognitive decline through a cascade of molecular and cellular interactions. The point is that companion dogs can provide a more precise diagnosis of cognitive deficit, due to the richer family environment and the easier detection of any change by owners. In our view, companion dogs represent a better model for deciphering the complex relationship between A β deposition, A β oligomers, neuronal activity and neuronal loss, glia participation, brain aging and the progressive cognitive dysfunction. Translating research into AD clinical outcomes may be easier with this dog paradigm, except in the case of initial research in pharmacological studies. Interestingly, the availability of companion dogs, despite their variety in breeds, sex, and life conditions, means there is no need to house a specific strain for years in controlled conditions.

Canine cognitive deficit and treatment studies

Until now, pharmacological studies of a variety of targets in canine with cognitive deficit have only partially improved cognitive decline and their development has required at some point the use of housed and no-housed dogs. Some of these treatments derive directly from AD pharmacology, and others, such as anti-A β immunotherapy, relate to innovative approaches in development. Influence of the dietary and environment quality is also considered to interfere with cognitive delay, as recently evidenced with the vitamin B supplementary treatment efficacy of AD patients [93].

Pharmacological treatments of CDS

1. L-deprenyl

L-deprenyl is currently used to treat Parkinson's disease and is also considered a potential treatment for AD. [94,95]. It selectively inhibits monoamine oxidase-B activity, increases the activity of several neuronal pathways and selectively induces catalase and superoxide dismutase activities in housed canine brain, conferring neuroprotection and prolonged cell survival. It was the first therapeutic agent approved (Anipril®) for use in canine CDS [35,96-98]. Although not all the mechanisms by which it produces cognitive improvement are clearly understood, enhancement of dopamine and other neurotransmitters in the cortex and

hippocampus is presumed to be a central aspect [99], improving neuronal impulse function and enhancing the learned cognitive function [96,100,101].

2. Nicergoline

Nicergoline is an ergoline derivative with a broad spectrum of action in many species, like rodents, dogs and humans: 1) as an α 1-adrenoceptor antagonist, it induces vasodilatation and increases arterial blood flow, 2) it enhances cholinergic and catecholaminergic neurotransmitter function, 3) it inhibits platelet aggregation, 4) it promotes metabolic activity, resulting in increased utilization of oxygen and glucose; and 5) it has neurotrophic and antioxidant properties due to its inhibition of lipid peroxidation, and it acts as a scavenger of free radicals [102,103]. Chronic treatment with nicergoline increases neuronal nitric oxide synthase expression in the cerebral cortex and basal ganglia, resulting in improved blood-brain perfusion [104]. Nicergoline is commercially available for the treatment of cognitive impairment in elderly dogs.

3. Propentofylline

Propentofylline is a neuroprotective glial cell modulator, which in preclinical studies has addressed some of the common pathological processes of AD and vascular dementia, including glial cell activation and increased production of cytokines, free radicals, and glutamate [105]. However, there is limited evidence that propentofylline might benefit cognition, global function and activities of daily living of patients with AD and/or vascular dementia [106]. Propentofylline is licensed for the treatment of dullness and lethargy in old dogs, in which it increases CNS oxygen supply without increasing glucose demand.

4. Adranafil

Adranafil increases the activity of the noradrenergic system and helps maintain alertness, wakefulness, attention and normal sleep-wake cycles by increasing daytime exploration and activity. Adranafil is also used in elderly humans to improve alertness. It is used off-label by individuals wishing to avoid fatigue, such as night workers or others to stay awake and alert for long periods of time. In housed dogs, oral administration of adranafil improves discrimination learning, increased locomotor activity, and causes a transient increase in directed sniffing [97].

5. A β Immunotherapy

This innovative approach is still under development as different vaccines for AD treatment, in order to accomplish pre-clinical and clinical objectives of efficacy and safety. This implies at least four stages: 1) the identification of an appropriate animal model to facilitate translation of the results to humans, 2) stimulation of a Th2 response modulating microglia cells to avoid cytotoxic activity, 3) CNS clearance of A β plaques

and of soluble A β monomers and oligomers, 4) proof of human cognitive improvement, and 5) proof of suitability in aged systems.

As noted above, certain limitations have restricted the use of transgenic models in the field of A β immunotherapy: owing to the complexity of AD and of the human immune response, it has not been possible to create mice that replicate these characteristics. In both these aspects dogs also presents many advantages. Their complete nuclear genome has a closer homology with human sequences than other AD models, except for the non-human primate model [14,107], and as noted above, the amino acid sequence of A β 1-40 and 1-42 is the same in canines and humans, and develops spontaneously as soluble mono- and oligomers, and A β plaques. In addition, canine and human aging present similar modifications of the innate immunity and cell-mediated changes, with significant reductions in lymphocytes, monocytes, granulocytes, T-cells, CD-8 cells and CD-4 cells [108,109]. As such, the total percentage of B-cells decreases while the percentage of T-cells increases. Both canines and humans also present the significant change from a predominance of naïve CD45RA⁺ to memory CD45RO⁺ phenotypes in CD4⁺ and CD8⁺ subsets [110], resulting in a reduction in the ability to respond to new antigens that correlates with the decreased naïve T-cells. Nonetheless, the ability to respond to recall antigens is maintained. In old dogs, as in aged humans, B-cell and T-cell functions are impaired due to a reduction of interleukin-2 (IL-2) receptor expression and IL-2 production [111,112], and with senescence the Th1 (pro-inflammatory):Th2 (anti-inflammatory) subpopulation ratio of blood lymphocytes also increases in both species [113,114]. Cytokines produced by these subpopulations regulate the immune response: while Th1 cells support CD-8 cellular function including cytokines such as Interferon γ (IFN γ), IL-12 and IL-15, Th2 cells provide humoral immune responses including IL-4, IL-5. Although in humans Th1 cells are more numerous than Th2 cells, these last ones produce more cytokines. Recently laboratories have been investigating the Th17 responses after any immunization [115]. The Th17 response has been associated with experimental autoimmune encephalitis in mice [116] and inflammatory diseases such as multiple sclerosis [117]. As in humans, dogs Th17 responses may relate to immunotherapy [118]. All these similarities between aging in humans and dogs full-fill the objectives mentioned above and make the aged canine a good model for investigating the safety and efficacy of human A β immunotherapy.

Some studies have already been carried out in housed beagle dogs [119] with no clear advantage. In our laboratory, an innovative active vaccine from different A β fibrillar fragments and compounds which induces Th2 responses has been developed in housed beagles and is being tested in companion CDS dogs. Our first results indicate at 2 months a significant and stable cognitive improvement in all treated animals, without any

side effect. The mechanisms involved in the amyloid clearance and their relationship with canine cognitive improvement are being studied.

Furthermore, A β immunotherapy in both aged housed and non-housed dogs make possible investigate the complex cellular and molecular mechanisms that would explain its effects on cognition. Experimental data from A β clearance highlight microglia participation [120-122] and that microglia clearance deficiency or reduced phagocytosis increase A β deposition in AD patients [123,123,124] and AD transgenic mice [125]. A β vaccine activates antigen presentation cells that in turn activate T cells through cytokines and peptide presentation. After a second signal, the soluble antigen presented by T cells binds to memory B cells for antibody production. Immunization produces Th1 (pro-inflammatory) or Th2 (anti-inflammatory) response in the peripheral immune system that affects CNS via a series of signals, such as cytokines, and activates A β clearance mechanisms. As shown in Fig.6, CNS A β removal (plaque and soluble forms) has been putatively attributed to five main mechanisms [126], with microglia and the CNS or periphery location of the anti-A β antibodies, the main parameters involved, either alone or in combination. Microglia removal action, implicated in the first and second mechanisms, is regulated by cytokines from the peripheral immune cells and/or from microglia modulation release. Then, microglia modulation will regulate synaptic processes and the blood-brain barrier (BBB) permeability which is altered in inflammatory processes. [127,128]. These five mechanisms illustrated in Fig.6 can be briefly described as follow:

- A) Microglial cell-mediated removal. Phagocytosis of the A β plaque by activated microglia and/or by CNS invasion of circulating macrophages-monocytes through scavenger receptors.
- B) Microglial cell-mediated removal by specific binding to the amyloid plaque
- C) Combined direct CNS interaction of antibodies and binding of microglia to the amyloid plaque
- D) Direct interaction of CNS antibodies to neutralize soluble toxic A β , resulting in a progressive reduction in the amyloid plaque
- E) The peripheral sink hypothesis, in which the peripheral A β antibodies and other plasma components such as albumin produce an efflux of CNS A β to the periphery in order to maintain the equilibrium between the central and peripheral compartments. The CNS amyloid load (plaque and soluble forms) is progressively reduced.

However, these five mechanisms cannot be considered mutually exclusive, as they depend on the type of immunotherapy used in each assay. It is generally accepted that the A β clearance response may differ according to the kind of immunotherapy used. For example, recently, RAGE specific modulation by a C-

terminal antibody has been shown to increase A β clearance in an in vitro BBB model, whereas the N-terminal antibody directly enhanced the basolateral-to-apical transcytosis of A β [129].

Dietary and complimentary therapy

Numerous studies have been performed in housed and no-housed aged dogs involving diets supplemented with a broad spectrum of antioxidants, and in some cases environmental enrichment [130,131]. Positive results have been recorded for memory, learning capacity and immune status [132-138].

A wide variety of complimentary therapies including nutraceuticals, herbal extracts and vitamins are currently marketed to improve or prevent canine cognitive deficits. For example, Senilife® (Innovet Italia S.l., Milano, Italy) is a combination of phosphatidylserine, *Gingko biloba*, pyridoxine, and Vitamin E; Novifit® tablets (Virbac, Forth Worth, USA) contains S-Adenosyl-L-Methionine-Tosylate Disulfate for the management of age-related mental impairment [139]. Geriactive® (Centaur Pharmacy, Guelph, USA) contains *Gingko biloba*, ginseng, bilberry and alpha-lipoic acid.

Environmental enrichment

More stimulating environments may facilitate treatment and improvement in a diverse variety of brain diseases such as AD, whereas lack of stimulation may impair cognitive development [140,141]. In dogs, the age-associated decline can be attenuated by physical exercise, social enrichment, and cognitive training, especially when combined with a complementary diet [132,133,142,143]. As a result, this cognitive enrichment has multiple effects and also improves functions not only related to regional neuronal loss or limited cellular parameters [134,144].

Conclusion

This review advocates the use of canines as a natural model to investigate the cascade of events in human aging and early stages of AD, as well as to validate innovative therapies, especially anti-amyloid immunotherapies, because of the similarities between human and canine immune systems. Together with a well-defined, complex behavior, dogs also have greater brain size and CSF availability than other mammals tested to date as AD animal models. Housed and non-housed dogs require specific behavior testing (which is more expensive and time-consuming in the case of housed animals), but both models are useful for other studies, bearing in mind the closer AD similarity of dogs with CDS and the difficulty to do complete pharmacological studies with them. Despite their variety in breed, sex and life conditions, the similar results obtained with housed and no-housed dogs indicate that many aspects can be investigated without the need to house a specific strain for years in controlled conditions. Therefore, dogs appear to be a simpler and practical model for assessment of geriatric subjects and pharmacology studies, they could avoid the use of non-human primates, and the results show that the quality of life of senior dogs has the potential to be improved.

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List of abbreviations

AD = Alzheimer's disease

A β = Amyloid Beta

ApoE= Apolipoprotein E

APP= Amyloid precursor protein

CSF= Cerebrospinal fluid

CDS= Cognitive dysfunction syndrome

ARCAD= Age-Related Cognitive and Affective Disorders

MMSE= Mini-mental state examination

LCD= Light cognitive deficit

YC= Young control

SCD= Severe cognitive deficit

MRI= Magnetic resonance imaging

GABA= Gamma aminobutyric acid

PV= Parvalbumin

CR= Calretinin

CB= Calbindin

Conflicts of interest

M Pugliese, MJ Rodríguez and N Mahy are co-authors of two patents to develop therapeutics for Alzheimer's disease and other disorders. The other authors declare no actual or potential conflicts of interest.

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Figure legends

Fig. 1 As a natural animal model of AD familiar dogs are a good alternative to housed dogs, besides their diversity of breed, sex and life conditions,

Fig.2 A) T₁- coronal MR images of different dogs with their rating of cerebral atrophy (0-4). Cerebral sulci, ventricular size, width of the temporal horns, and hippocampal height were evaluated (0 or 1) for assessment of cerebral atrophy. Score 0 (each parameter 0); score 1 (ventricular size 1); score 2 (ventricular size 1 and cerebral sulci 1); score 3 (ventricular size 1, cerebral sulci 1 and height of hippocampus 1); score 4 (ventricular size 1, cerebral sulci 1, height of hippocampus 1 and width of temporal horn 1). B) T₁-axial MR images of different dogs with their rating of cerebral atrophy (0-4). Score 0 (each parameter 0); score 2 (cerebral sulci 1 and width of temporal horn 1); score 4 (ventricular size 1, cerebral sulci 1, height of hippocampus 1 and width of temporal horn 1).

Fig.3 A β peptide participates directly in mitochondrial dysfunction and reactive oxygen species formation present in neurodegeneration leading to DNA and protein oxidation and lipid peroxidation

Fig.4 Representative micrographs of A β immunoreactivity in the prefrontal cortex of three dogs visualized with anti-A β 8-17 antibody (A-C). **A**) In stage I- II A β deposition is localized in the deep layers of the cortex (V-VI) and showed a diffuse and cloud-like aspect. **B**) In stage III A β deposition is localized in more superficial cortical layers (I-III) and consisted of more dense diffuse plaques, similar in morphological appearance to human senile plaques. **C**) In stage IV more compact plaques are progressively extended throughout all cortical layers.

D) Represents phospho-tau Tau Thr181 immunofluorescence (green) and A β plaques (red) in one CDS dog (20 years old). **E**) Represents phospho-tau Ser396 (green) and A β plaques (red) in another CDS (16 years old). In none of them positive neurite surrounding A β plaques were observed (A-C) =20 μ m. Bar (D-E)= 20 μ m. The sections were counterstained with TO-PRO-3 (blue) in D and E.

Fig.5 The proximity (μm) of astrocytes to the amyloid plaque (AP) is different when it expressed S100 β . Immunoreactive GFAP astrocytes are in III and IV stage plaques. S100 β (S100 β -IR, green line) astrocytes are closer in stage IV. (GFAP-IR blue square, negative S100 β -IR)

Fig.6 Proposed clearance mechanisms induced by an active A β immunization. Microglia cell mediates A β plaque phagocytosis by activated microglia and/or by central CNS invasion of circulating phagocytic cells (A,B), antibodies interact directly with the plaque, and for some authors also with microglia to participate in the disaggregation of the plaque (C), direct interaction of CNS antibodies to neutralize soluble A β (D), peripheral sink hypothesis in which peripheral A β antibodies and other plasma components such as albumin produce an efflux of A β from CNS to periphery (E).

Figures

Fig.1



Varitety of dogs

Fig.2

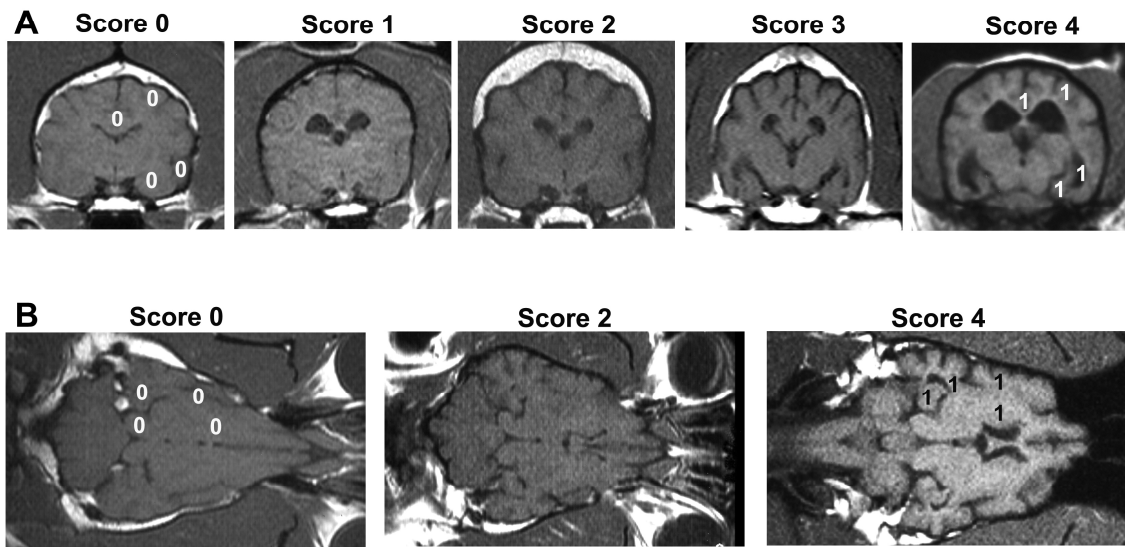


Fig.3

Free radical formation and oxidative damage

Endogenous oxidants

- Mitochondrias (Abeta)
- Peroxisomes
- Lipoxygenases
- NADPH oxidases
- Cytochrome P450

Exogenous oxidants

- UV radiations
- Ionizing radiations
- Chemoterapics
- Antibiotics
- Environmental toxins

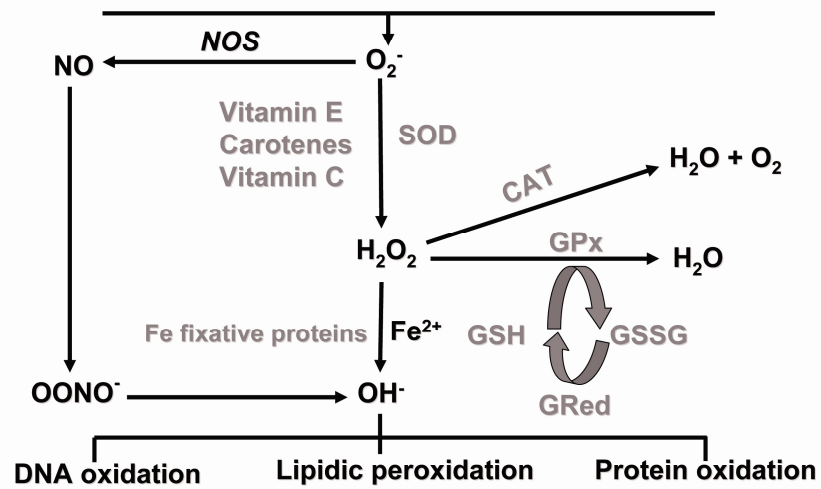


Fig.4

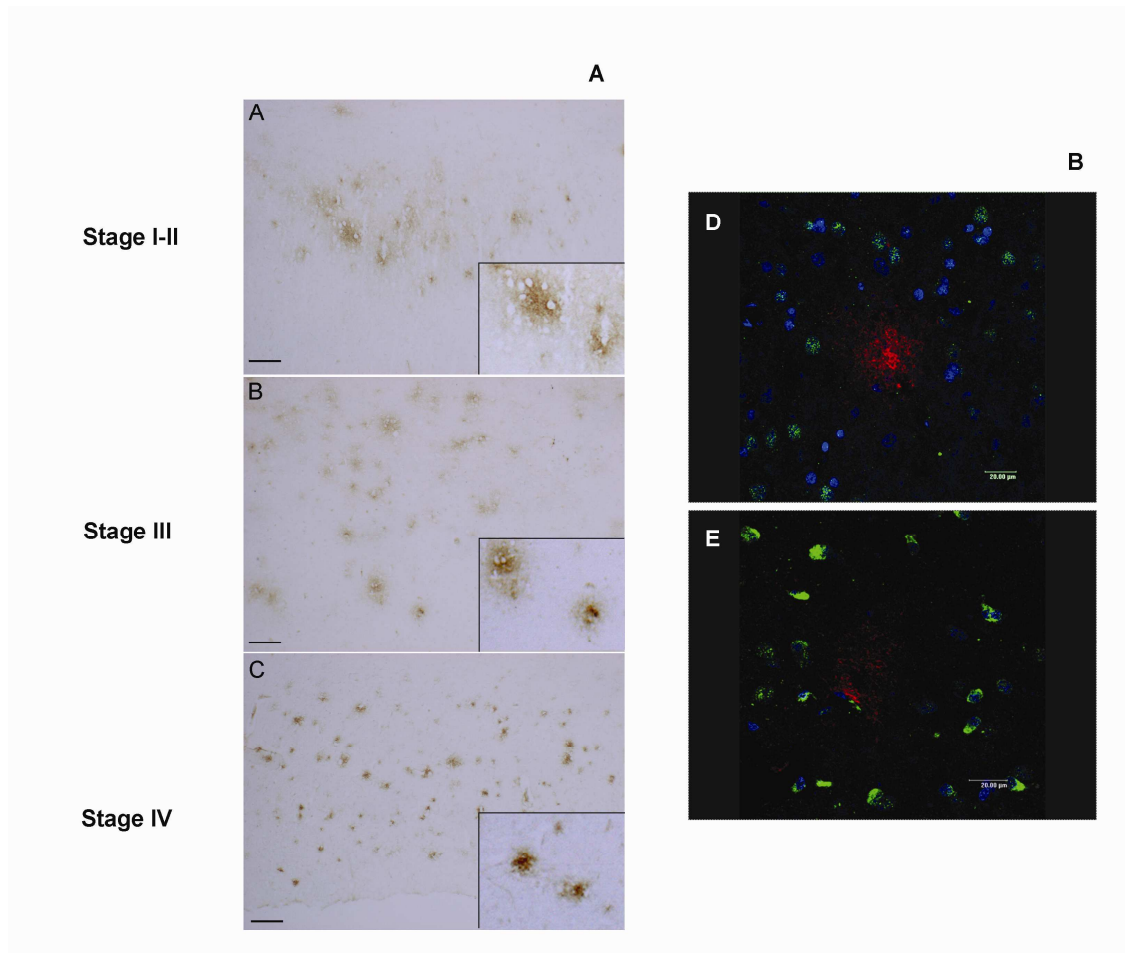


Fig.5

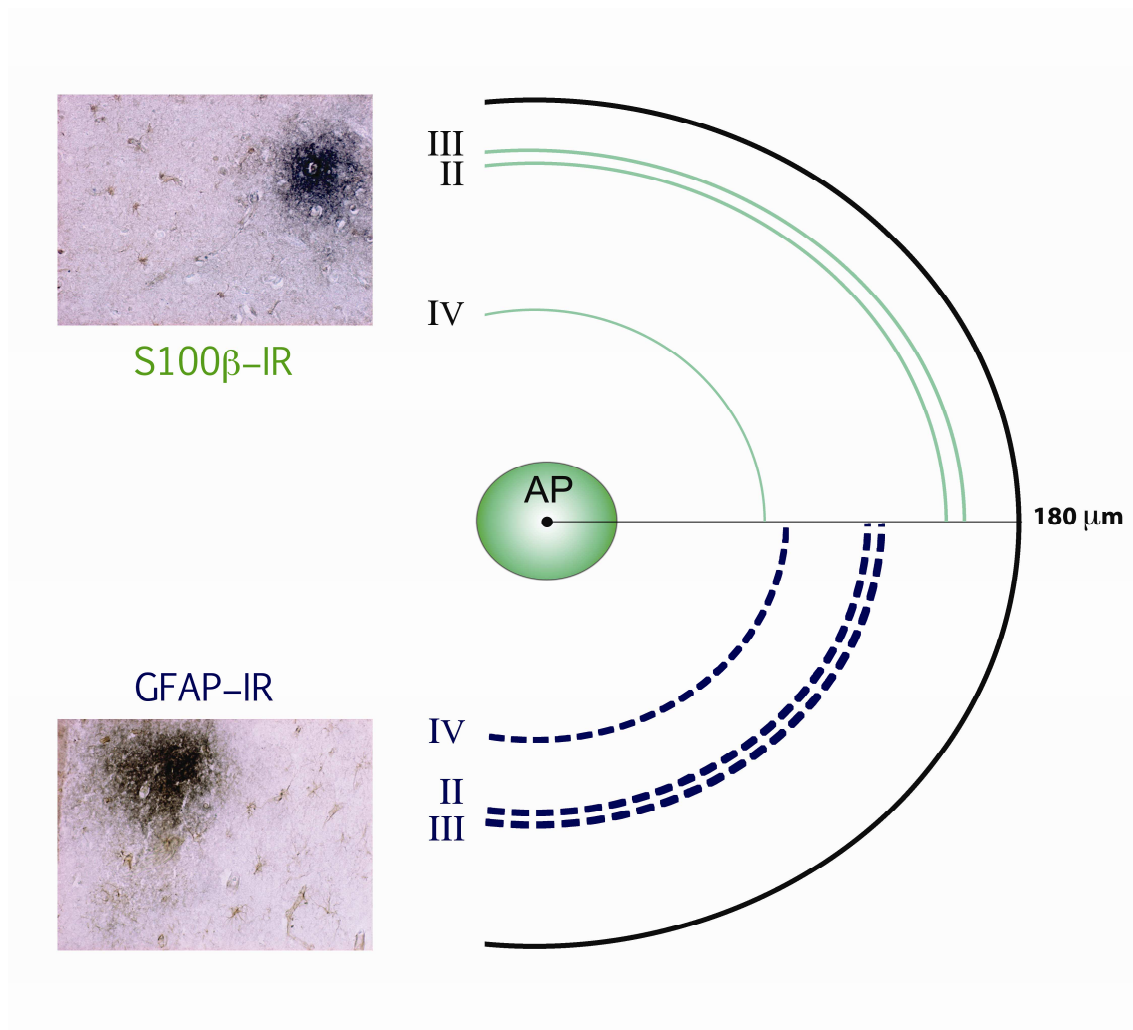


Fig.6

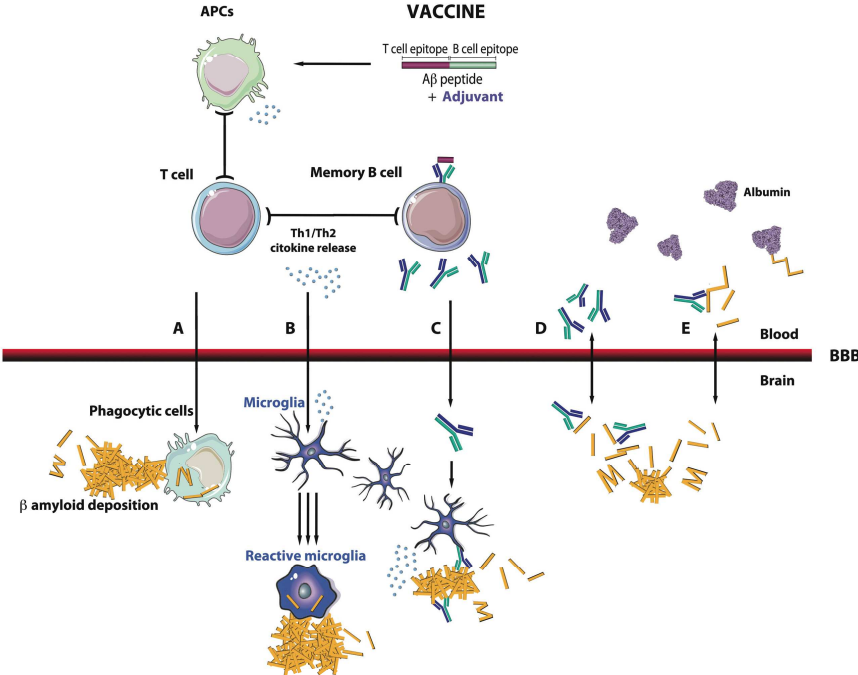


Table 1: Characteristics of natural and induced models of Alzheimer's disease

Natural models of Alzheimer's disease			
	Cognitive aspects	Neuropathology	Limitations
Aged primates [145]	Learning, memory and cognition impairment	Diffuse and neuritic A β plaques Soluble A β Phospho-tau tangles Dsyrophic neurites Neuronal loss Gliosis	Elevated costs studies Long-term housing
Non-housed CDS dog [68,91]	Learning, memory and cognition impairment* Behavioral changes	Diffuse A β plaques Soluble A β Hyperphosphorylated Tau Neuronal loss Gliosis	Consent by the owner Lower costs studies Facilities not required
Housed aged dogs [37]	Learning, memory and cognition impairment	Diffuse A β plaques Soluble A β Hyperphosphorylated Tau Neuronal loss Gliosis	Time consuming training for tasks learning Elevated costs studies Long-term housing
Induced models of Alzheimer's disease			
Fruit fly A β 40 or 42 fly [146] Tau-pathology fly [147]	Associative learning and memory impairment	Amyloid deposits Primary tauopathy features Neuronal loss	No parallelism between fly brain and mammalian brain
Rabbit Hypercholesterolemia rabbit model [148]	Cognitive impairment	A β plaques Gliosis Neuronal loss	Hypercholesterolemia model Limitation in learning, memory and cognition capacities
Rat Intraventricular Abeta infusion [149]	Mild impairment	A β plaques Gliosis	Aggregates in the pump No evidences of learning, memory, cognitive impairment
Transgenic rats Cited in review [145]		A β plaques Gliosis	Low efficacy in the genome rats manipulations No evidence of learning, memory, cognitive impairment
Mouse SAMP8 [150]	Mild cognitive impairment model	Oxidative damage	Mild cognitive impairment model Time consuming training for tasks learning
Trangenic mice Tg 2576 [8] PSAPP [151] 3xtg [152]	Spatial cognitive and learning impairment	Neuritic and diffuse plaques Gliosis Neuritic dystrophy Hyperphosphorylated Tau	Time consuming training for tasks learning Elevated costs studies

Table 2: Cognitive test for the valuation of cognitive deficit in companion dogs

Cognitive test					
Items	Score	Items	Score	Items	Score
(1) Walking Normal Trudging Abnormal, one direction, circling	1 3 5	(7) Life rhythm Normal Rest and sleep over during the day Switches between insomnia & hypersomnia Restless at bedtime	1 2 3 5	(13) Auto-stimulatory behavior Normal Alteration seeking licking and nibbling Stereotyped nibbling, tail chasing Repeated movements of licking, straching and nibbling	1 2 3 5
(2) Eating Normal Regurgitation and re-ingestion Anorexia Hyperphagia/tachyphagia	1 2 3 5	(8) Play behaviour Normal Increase Decrease	1 3 5	(14) Learned social behaviour Normal (unchanged) Bites without warning Does not submit itself when rebuked Steal and retains the stolen objects	1 2 3 5
(3) Posture/ emotional of expression Normal Decrease of body language Abnormal, loss of body language	1 3 5	(9) Exploratory behaviour Normal Decrease (disorientation expectance posture, etc) Increase (obsessive exploration of the same place, including oral exploration)	1 3 5	(15) Adaptative capabilities Normal Retreats from novel situation Unable to accept changes in routine Looks indifferent to changes	1 2 3 5
(4) Barking Normal Monotonous and loud Barking throughout night or at unusual object	1 3 5	(10) Self-control Normal Difficulties to calm down after a stressful event Alternate periods of hyperactivity and indifference Tend to generalize aversive experience	1 2 3 5	(16) Interaction with other animals or with owners Normal Decreased (loss of relationship with humans or other animals) Complete loss relationship with owners	1 3 5
(5) Drinking Normal Champing at water without swallowing Polydipsia	1 3 4	(11) Aggressive behavior Absence (never showed) Aggressiveness for fear Aggressiveness for irritability	1 3 5		
(6) Elimination behavior Normal Defecate and urinate at home: occasionally in a small scattered amount Loss control of sphinters: defecate and urinate outside of sleeping area Loss control of sphinters: defecate and urinate inside of sleeping area	1 3 4 5	(13) Learned specific behavior Normal (unchanged) Random responses Decreased response	1 3 5		

Total score in 21 items test; <23=normal; 23-33=Light cognitive deficits; >33=Severe cognitive deficits.

Total score in the 9 items test (indicated in bold): <12= normal; 12-26= I; >26=severe cognitive deficits

Adapted from Pugliese et al. 2005

Table 3: Relation between A β plaques in prefrontal cortex and behavioral tests of companion and housed dogs

Relation between Aβ plaques in prefrontal cortex and behavioral tests			
Companion dogs (Different breeds and sex)			
<i>Age (years)</i>	<i>Plaque density (Plaques/mm²)</i>	<i>Plaque stage maturation</i>	<i>Cognitive score test (using 9 item test)</i>
1-6(n=4)	0)		9-11(YC)
8-15 (n=5)	12.8 \pm 7.75	I-II	13-21(LCD)
14-20 (n=7)	26.85 \pm 12.10	II-IV	27-40(SCD)
Housed dogs (Beagle breed and different sex)			
<i>Age (years)</i>	<i>Plaque occupancy (% area occupied by Aβ immunostaining)</i>	<i>Cognitive tasks</i>	
4.3-15.3 (n=7)	0-0.9	- Object discrimination learning - Reversal learning	
8.4-15.3 (n=13)	0-21.3		

Companion dogs classified into 3 groups depending of their age and present a correlation between age, the abundance of plaques, their stage of maturation and the cognitive test score

YC= young control group; LCD= Light cognitive deficit group; SCD= Severe cognitive group.

Housed dogs classified into young and aged animals present a correlation with amyloid load, age and number of errors in two cognitive tasks

Adapted from *Pugliese et al. 2005 and Head et al. 1997*